PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 31/195, A61L 27/00, 25/00 | A1

(11) International Publication Number: WO 00/06150

(43) International Publication Date: 10 February 2000 (10.02.00)

(21) International Application Number: PCT/EP98/04713

(22) International Filing Date: 28 July 1998 (28.07.98)

(71) Applicants *(for all designated States except US)*: SYNERGEN AG [CH/CH]; Alte Steinhauserstrasse 35, CH–6330 Cham (CH). AO–FORSCHUNGSINSTITUT DAVOS [CH/CH]; Clavadellerstrasse, CH–7270 Davos Platz (CH).

(72) Inventors; and

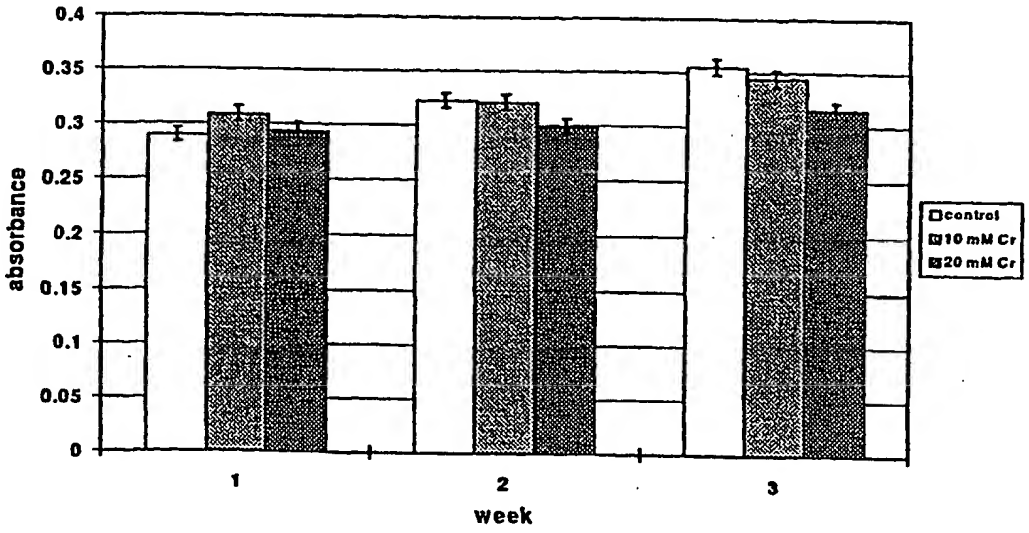
(75) Inventors/Applicants *(for US only)*: WALLIMANN, Theo [CH/CH]; Schürmattstrasse 23, CH–8963 Kindhausen (CH). GERBER, Isabel [CH/CH]; Dorfstrasse 9, CH–7260 Davos Dorf (CH).

(74) Agent: LUSUARDI, Werther; Dr. Lusuardi AG, Kreuzbühlstrasse 8, CH–8008 Zürich (CH).

(81) Designated States: CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
*With international search report.*

(54) Title: USE OF CREATINE COMPOUNDS FOR TREATMENT OF BONE OR CARTILAGE CELLS AND TISSUES




(57) Abstract

The method, composition and use of the composition for healing defects in bone or cartilage tissue in animals and humans caused by trauma or surgery is disclosed. The method comprises administration of creatine compounds including analogues or pharmaceutically acceptable salts thereof. Treatment in accordance with this method speeds–up time for and improves the process of healing of defects in bone or cartilage tissue in animals and humans caused by trauma or surgery including acceptance and bonding of artificial implants. The treatment with creatine compounds can be therapeutic for diseased patients, preventive for healthy people as well as geriatric for elderly people.

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| AL | Albania | ES | Spain | LS | Lesotho | SI | Slovenia |
| AM | Armenia | FI | Finland | LT | Lithuania | SK | Slovakia |
| AT | Austria | FR | France | LU | Luxembourg | SN | Senegal |
| AU | Australia | GA | Gabon | LV | Latvia | SZ | Swaziland |
| AZ | Azerbaijan | GB | United Kingdom | MC | Monaco | TD | Chad |
| BA | Bosnia and Herzegovina | GE | Georgia | MD | Republic of Moldova | TG | Togo |
| BB | Barbados | GH | Ghana | MG | Madagascar | TJ | Tajikistan |
| BE | Belgium | GN | Guinea | MK | The former Yugoslav Republic of Macedonia | TM | Turkmenistan |
| BF | Burkina Faso | GR | Greece | | | TR | Turkey |
| BG | Bulgaria | HU | Hungary | ML | Mali | TT | Trinidad and Tobago |
| BJ | Benin | IE | Ireland | MN | Mongolia | UA | Ukraine |
| BR | Brazil | IL | Israel | MR | Mauritania | UG | Uganda |
| BY | Belarus | IS | Iceland | MW | Malawi | US | United States of America |
| CA | Canada | IT | Italy | MX | Mexico | UZ | Uzbekistan |
| CF | Central African Republic | JP | Japan | NE | Niger | VN | Viet Nam |
| CG | Congo | KE | Kenya | NL | Netherlands | YU | Yugoslavia |
| CH | Switzerland | KG | Kyrgyzstan | NO | Norway | ZW | Zimbabwe |
| CI | Côte d'Ivoire | KP | Democratic People's Republic of Korea | NZ | New Zealand | | |
| CM | Cameroon | | | PL | Poland | | |
| CN | China | KR | Republic of Korea | PT | Portugal | | |
| CU | Cuba | KZ | Kazakstan | RO | Romania | | |
| CZ | Czech Republic | LC | Saint Lucia | RU | Russian Federation | | |
| DE | Germany | LI | Liechtenstein | SD | Sudan | | |
| DK | Denmark | LK | Sri Lanka | SE | Sweden | | |
| EE | Estonia | LR | Liberia | SG | Singapore | | |

# Use of creatine compounds for treatment of bone or cartilage cells and tissues.

This invention concerns the use of creatine compounds in accordance with the pre-characterising portion of claim 1, a method for accelerating healing in an animal or human having a defect in bone or cartilage tissue in accordance with the pre-characterising portion of claim 40, a composition useful for the treatment of defects in bone or cartilage tissue in accordance with the pre-characterising portion of claim 41 and the use of creatine kinase in accordance with the pre-characterising portion of claim 43.

The creatine compounds may be incorporated in three dimensional constructs of osteoblasts, chondrocytes or mesenchymal stem cells designed for tissue engineering of said bone or cartilage defects.

Further the creatine compounds may be used for improving acceptance and osseous integration of bone implants.

Creatine is a compound which is naturally occurring in the human body and is found in mammalian brain and other excitable tissues, such as skeletal muscle, heart and retina. It's phosphorylated form, creatine phosphate is also found in the same organs and is the product of the creatine kinase reaction utilizing creatine as a substrate. Creatine and creatine phosphate can be synthesized relatively easy and are believed to be non-toxic in mammals.

The use of creatine and analogues thereof for the treatment of diseases of the nervous system have already been described in WO-A-96/14063 AVICENA GROUP INC. (US priority application serial number 08/336,388) the disclosure of which is hereby incorporated in the present patent application.

However, nowhere has the use of creatine kinase or creatine compounds for the treatment of bone and cartilage cells or tissues been specifically disclosed or advocated for the treatment of bone and cartilage in health and disease.

The present invention as claimed provides for new use of creatine kinase and creatine compounds (which modulate one or more of the structural or functional components of the creatine kinase /creatine phosphate system) as a therapeutic agents. More particularly, the present invention provides methods of:

a) treatment of bone or cartilage diseases
(e.g. osteoporosis, osteoarthritis or periodontitis);

b) promoting growth or mineralisation of bone or cartilage cells and tissues;

c) conservative or operative treatments of bone fractures or bone defects;

d) applying bone or cartilage grafts to bone or cartilage fractures or defects;

e) tissue engineering by extracorporeal culture of bone or cartilage forming cells (obtained from a healthy individual or particular patient) in the presence of creatine to form a three-dimensional cell assembly which can be transferred in a subsequent step to a specific location having a bone or cartilage defect of the same particular patient;

f) metabolic engineering of bone and cartilage cells by transfection with DNA coding for creatine kinase in order to make said cells overexpress creatine kinase and thus together with creatine improve, stimulate and stabilize the physiological function of said cells and tissues for reimplantation into patients as outlined in section e).

In all these applications of creatine the essential function of creatine (Cr) is its ability to act as an energy source and regulator of cellular energy metabolism, as well as a cell protective agent against metabolic stress. In addition, creatine has surprisingly shown to exert a protective effect on early events of programmed cell death or apoptosis. These effects are all mediated by creatine kinase.

The surprising effect of the creatine compounds on bone and cartilage cells and tissues has been to speed-up time for and improve the process of healing wounds in bone or cartilage tissue caused by trauma or surgery, including bone fractures and the acceptance and bonding of artificial implants. The treatment with creatine compounds can be therapeutic for diseased patients, preventive for healthy people as well as geriatric for elderly people. A variety of creatine compounds may be used in connection with the invention, in particular those selected from the group of creatine, creatine phosphate, creatine pyruvate and cyclocreatine.

The creatine compounds may be in the form of a pharmacologically acceptable salt or combined with an adjuvant or other pharmaceutical agent effective to treat bone and/or cartilage cells. Compounds useful in the present invention are creatine compounds which modulate the creatine kinase system.

The present invention also provides pharmaceutical compositions containing creatine compounds in combination with a pharmaceutical acceptable carrier. Such carriers are disclosed e.g. in

"Principles of Tissue Engineering, Chapter 19: Biodegradable Polymers for Tissue Engineering, J.M.Pachence and J. Kohn, 1997, pag. 274-293; and in

"Der Orthopäde, Bone replacement materials, J.M.Rueger, 2-1998, pag 73-79;

the disclosure of which is hereby incorporated in the present patent application.

The compositions according to the invention may be administered orally, in form of granulates or in a sustained release formulation. By sustained release is meant a formulation in which the composition becomes biologically available to the patient at a measured rate over a prolonged period. Such compositions are well known in the art.

The main route of Cr biosynthesis in mammals involves the formation of guanidinoacetate in the kidney, its transport through the blood and its methylation to Cr in the liver. Cr, exported from the liver and transported again through the blood, may then be taken up by the Cr-requiring tissues via the creatine transporter protein. When mammalian cells are cultured, creatine is available only in the amounts present in the serum added, which contains 0,05 - 0,10 mM Cr.

The enzyme creatine kinase (CK) plays a key role in the energy metabolism of cells that have intermittently high and fluctuating energy requirements. CK isoenzymes are found predominantly in skeletal and cardiac muscle, but also in spermatozoa (vertebrate and sea urchin sperm), electrocytes of the electric organ of electric fish, photoreceptor cells of the retina and the lens of the eye, brain (glial and neuronal cells of the cerebellum, glomerular structures of the cerebellum, neurones), the uterus and placenta, intestinal brush border epithelial cells and endothelial cells, kidney and rectal salt

gland, adipose tissue, pancreas, thymus, thyroid and liver, cartilage and bone, macrophages, blood platelets, as well as in certain malignant tumours and cancer cells.

The reaction catalyzed by CKs, the reversible transfer of the phosphoryl group from phosphocreatine (PCr) to ADP, allows regeneration of the key cellular energy carrying molecule ATP. Cells contain a number of different CK isoforms, which are not evenly distributed in cells. They are compartmentalized in an isoform-specific fashion the two isoforms M-CK and B-CK are cytosolic, and two of the isoforms Mia-CK and Mib-CK are specifically mitochondrial. These various isoforms of CK are thought to constitute an intricate energy buffering and transport system, connecting sites of high energy phosphate production (by glycolysis and oxidative phosphorylation) to sites of energy consumption (ATPases).

The mitochondrial CK isoforms (Mi-CK) are located along the outer surface of the entire inner membrane and also at sites where the inner and outer membranes are in close proximity. At these latter sites, Mi-CK can directly use intra-mitochondrially produced ATP to generate PCr, which is exported to the cytosol where it serves as an easily diffusible, energy-storage metabolite. In contrast to the cytosolic CK isoforms, which are dimeric, Mi-CK, forms highly symmetrical, cube-like octamers (Schnyder et al. 1991) that can bind to the periphery of lipid membranes. Most importantly Mi-CK can mediate contact-site formation between the inner and the outer mitochondrial membranes and in addition, Mi-CK is functionally coupled to oxidative phosphorylation by the adenine nucleotide transporter which catalyzes ATP/ADP

antiport across the inner membrane. Net PCr production can be stimulated by addition of extra-mitochondrial Cr, even in the presence of external ATP-regeneration systems and ATP sinks.

**Creatine and phosphocreatine in cartilage**

Resting and hypertrophied cartilage both contain PCr. However, the distribution of PCr varies in the different zones of the cartilage. The highest content of Cr is in the resting cartilage. The other zones have similar amounts of Cr. On the other hand, the highest amount of PCr is found in the proliferative zone of cartilage with lower concentration in resting and hypertrophic cartilage. In calcified cartilage-bone, PCr is indetectable.

Experimental studies show that external addition of PCr promotes cartilage mineralization in organ and cell culture. The deposition of calcium in the cartilage matrix of the epiphysis of cultured embryonic chick femora is accelerated by the addition of very crude preparations of PCr and Cr at 0,1 mM in chick embryo extract with 20 % horse serum. Mineralization in differentiating chick limb bud mesenchymal cells in micromass cultures is promoted by the addition of 1 and 2 mM ATP or 2 mM PCr. The formed mineralized cartilage matrix is similar to that *in ovo*. The addition of ATP or PCr does not alter the rate of cell proliferation, the rate of matrix synthesis, the mean crystallite length or the rate of mineral deposition, when contrasted with cultures supplemented with inorganic phosphate. The ultrastructure of the cultured cells in the presence of 4 mM inorganic phosphate (Pi), 1 - 2 mM ATP or 2 mM PCr are similar at

day 14 and 21. There are differentiated chondrocytes within the nodule containing hypertrophied and degenerating cartilage. At the edge of the nodule the cartilaginous matrix containing type II collagen, proteoglycans and matrix vesicles is surrounded by undifferentiated cells and type I collagen. ATP, PCr or Pi do increase the mineral to matrix ratio around the edge of the micromass but not in the centre of the cartilage nodule (low mineral to matrix ratio). There is no difference in the pattern of mineralization due to Pi, ATP or PCr.

Reduction of the Cr uptake by feeding rats with beta-guanidinopropionate (GPA) results in marked abnormalities in the epiphyseal growth plate of the rats. The zone of calcified cartilage is wider and extends into the metaphysis. The hypertrophic chondrocytes are vacuolated and poorly columnated and mineralization is less abundant and occurs also in the transverse septa. Vascular invasion is poor. There is a reduction in the osteoid formation. GPA interferes with the synthesis of pro-a type II and type X collagen in cultured chondrocytes.

**Creatine and phosphocreatine in bone**

PCr increases the alkaline phosphatase (ALP) activity in SaOS-2 cells. The perichondral ossification in the diaphysis of cultured embryonic chick femora is accelerated by the addition of PCr and Cr preparations at 0.1 mM to chick embryo extract with 20% horse serum.

**Creatine kinase in cartilage**

The level of CK activity is correlated to the chondrocyte maturation in the epiphysis and in the rib. There is an increase in CK activity from the resting-proliferative cartilage to the hypertrophic cartilage (6 fold) and the calcified cartilage-bone zone (17 fold). In resting and proliferating cartilage the predominant CK isoform is MM. M-CK is 1/3 to 1/5 of those in skeletal muscle (160'000 ng/mg protein) and the amount is independent of the age. In hypertrophic cartilage the MB-CK and BB-CK isoforms are predominant and B-CK is 30 - 47 fold higher than in skeletal muscle (60 ng/mg protein and B-CK shows a significant decrease with advancing age.

CK activity seems to be required for matrix synthesis and mineralization of the enchondral growth cartilage and chondrocytes in culture undergoing hypertrophy show an increase in the CK activity. CK activity peaks in the cartilage in rats of peripubertal age.

CK activity in the cartilage is stimulated by growth hormone (GH), by insuline-like growth factor 1 (IGF-I), by metabolite of vitamine D [$24R,25(OH)_2D_3$] in normal rats and in vitamin D-deficient rats, by PTH, by protease-resistant variants of parathyroide hormone (PTH), by 17b-estradiol in normal rats and in ovariectomized rats. Stimulation of BB-CK activity is followed by a parallel increase in DNA synthesis.

In rachitic cartilage the profile of CK is similar but the values in the hypertrophic and also in the calcified cartilage are lower than in the normal cartilage.

**Creatine kinase in bone**

In embryonic chick bone there is BB-CK along with some MB and MM-CK activity. During early facial development there is a prominent anaerobic metabolism in the facial processes, BB-CK is present from the $9^{th}$ embryonic day, and during $10^{th}$ -$15^{th}$ days MB-CK and MM-CK develop. The amount of bone produced during hetereotropic bone formation by implantation of BMP into muscle of rats shows an almost parallel relationship with the levels of S-100b protein, B-CK, hyaluronic acid and chondroitin sulphate and the activity of ALP. B-CK expression is modulated by transcriptional and posttranscriptional mechanisms during differentiation of osteoblastic cells. Enhanced activity of membrane pumps and changes in the cytoskeleton are related to the formation of extracellular matrix and mineralization.

In bone, similar to cartilage, BB-CK is also experimentally increased both *in vitro* and *in vivo* by IGF-I; by $1,25(OH)_2D_3$; by PTH; by protease-resistant variants of PTH; by $PGE_2$; by 17b-estradiol ($E_2$). Furthermore, the stimulation of the bone cell energy metabolism by 17b-estradiol ($E_2$) and testosterone is sex specific, as shown in diaphyseal bone of weanling rats but not in epiphyseal cartilage. $E_2$ causes a 70-200% increase in CK activity *in vivo* and *in vitro* in ROS 17/2.8, in MC3T3-E1 cells and foetal rat calvaria cells and a 40% increase in rat epiphyseal cartilage cells. The stimulation of $E_2$ is dose- and time-dependent. Ovariectomized rats between 1-4 weeks after surgery show a stimulation of CK by $E_2$, 24h after injection. Both the basal and stimulated activity of CK is higher in the diaphysis and

epiphysis than in the uterus. The effect of $E_2$ *in vivo* and in chondroblasts and osteoblasts *in vitro* is inhibited by high levels of the anti-oestrogen tamoxifen which by itself in high concentration shows stimulatory effects. Furthermore, gonadectomy causes a loss of the sex-specific response of diaphyseal bone to steroid hormones. CK activity peaks in diaphyseal bone and cartilage in rats of peripubertal age. Patients with autosomal dominant osteopetrosis Type II have an elevated level of BB-CK but patients with other sclerosing bone diseases do not show such an elevation in BB-CK.

For adult humans (70 kg) the daily dosage of chemically pure creatine monohydrate is in the range of 0,1 to 20,0 g per day, preferably with a loading phase of 4 times 4-6 g per day for the first 8-14 days and a maintenance dosage of 2-4 g per day for another 3 months with an interruption of the supplementation scheme for one month thereafter.
To improve bioavailability, chemically pure creatine monohydrate can be mixed with carbohydrates like maltodextrins and/or dextrose and others.

The various features of novelty which characterize the invention are pointed out with particularity in the claims annexed to and forming part of this disclosure. For the better understanding of the invention, its operating advantages and specific objects attained by its use, reference should be had to

the accompanying drawings, examples and descriptive matter in which are illustrated and described preferred embodiments of the invention.

In the drawings:

Figure 1 is a graph showing Viability (NR) of monolayer osteoblast cell cultures at 1, 2 and 3 weeks in the absence (control) and presence of either 10 mM or 20 mM creatine in the medium. Least squares means ± 1,96 standard error;

Figure 2 is a graph showing metabolic activity (MTT) of monolayer osteoblast cell cultures at 1, 2, and 3 weeks in the absence (control) and presence of either 10 mM or 20 mM creatine in the medium. Least squares means ± 1,96 standard error;

Figure 3 is a graph showing mineralization of monolayer osteoblast cell culture at 2 and 3 weeks in the absence (control) and presence of either 10 mM or 20 mM creatine in the medium. Least squares means ± 1,96 standard error;

Figure 4 is a graph showing mineralization of micromass osteoblast cell culture at 2 and 3 weeks in the absence (control) and presence of either 10 mM or 20 mM creatine in the medium. Least squares means ± 1,96 standard error; and

Figure 5 is a graph showing embryonic rat femora wet weight after 3 weeks in organ culture, with and without 10 mM or 20 mM creatine. Least squared means ± 1,96 standard error.

**Aim of the experimental work**

The effects of supplementation with creatine (Cr) and beta-guanidinopropionic acid (GPA; a Cr analogue and competitor of Cr uptake into the cell) on the differentiation of osteoblasts and chondrocytes *in vitro* were determined. The parameters investigated were viability (based on the physical uptake of neutral red and the metabolic activity), histochemical ALP activity and degree of mineralization, as well as the TEM ultrastructure.

**Methods**

**Cell culture**

This isolation technique is based on the ability of osteoblasts to migrate from bone onto a substratum. Parietal and frontal calvariae (4 per animal) were aseptically explanted from 6 day

old IcoIbm rats. The calvariae were placed in Tyrode's balanced salt solution, calcium and magnesium free (TBSS). The periosteum was enzymatically removed with 0,05 % trypsin and 0,02 % collagenase A (0,76 U/mg) dissolved in TBSS (40 calvaria/20 ml). The calvariae were shaken for 70 minutes in a waterbath at 37°C. They were washed with TBSS and then transferred to 60 mm culture dishes (40 calvariae/dish) containing 5 ml of 0,02 % collagenase A (0,76 U/mg) in culture medium $BGJ_b$ Fitton-Jackson modification and placed in the incubator for 4 hours. Afterwards the calvariae were washed with culture medium B supplemented with 10 % foetal calf serum (FCS). The calvariae were transferred into 60 mm culture dishes (4 frontal and 4 parietal/dish). The growth medium supplemented with 10 % FCS and 50 µg/ml ascorbate was completely changed every 48 h. The culture was kept for 3 weeks.

After 3 weeks the migrated cells were harvested. The dish was washed with TBSS and 5 ml of TBSS containing 0,05 % trypsin and 0,02 % collagenase A (0,76 U/mg) was added. After 1 hour in the incubator the dish was washed with culture medium $BGJ_b$ supplemented with 10 % FCS. The dishes containing the calvariae and cells were rinsed with serum containing media $BGJ_b$. The cells obtained were filtered through a 40 µm nylon mesh to remove bone debris and cell aggregates. The suspended cells were centrifuged at 600 g for 5 minutes. The cell pellet was resuspended in serum containing medium $BGJ_b$ and centrifuged. The viability of the resuspended cells was examined by the 'dye exclusion' of 0,4 % trypan blue and the cells counted in a haemocytometer. The

inoculation densities were $2 \cdot 10^5$ /10 $cm^2$ for monolayer and $2 \cdot 10^5$/30 µl for micromasses. The micromass cultures were kept for 1 h in the incubator before 2 ml growth medium was added.

**Organ cultures**

**Calvariae with periosteum**

Parietal and frontal calvariae (4 per animal) were aseptically explanted from 6 day old IcoIbm rats. The calvariae were washed thoroughly with TBSS, then transferred into 60 mm culture dishes (4 frontal and 4 parietal/dish) containing growth medium $BGJ_b$ supplemented with 50 µg/ml ascorbate either serum-free or with 10% FCS. The medium was changed completely every 48 h. The culture was kept for 3 weeks and then processed for histology.

**'Denuded' calvariae**

The periosteum was enzymatically removed with 0,05 % trypsin and 0,02 % collagenase A (0,76 U/mg) dissolved in TBSS (40 calvariae/ 20 ml). The calvariae were shaken for 70 minutes in a water bath at 37°C. They were washed with TBSS. The calvariae were then transferred to 60 mm culture dishes (40 calvariae/dish) containing 5 ml of 0,02 % collagenase A (0,76 U/mg) in culture medium $BGJ_b$ and placed in the incubator for 4 hours. Afterwards the calvariae were washed with culture medium $BGJ_b$ supplemented with 10 % FCS. The calvariae were transferred into 60 mm culture dishes (4 frontal and 4 parietal/dish). The growth medium $BGJ_b$ supplemented with 50 µg/ml ascorbate was either used

serum-free or with 10% FCS and was completely changed every 48 h. To study the effect of FCS the cultures were kept for 3 weeks and then processed for histology.

To study the bone regeneration capacity of calvariae they were kept as long-term cultures for 6, 9, 12, 15 weeks in growth medium with 10 % FCS. Every 3 weeks these calvariae were transferred into a fresh culture dish. At the endpoint the calvariae were processed for histology.

**Embryonic long bones**

The rats were sacrificed on the $17^{th}$-$18^{th}$ day of pregnancy. The embryos were aseptically removed from the uterus and both femora were carefully dissected free into sterile TBSS under the stereo-microscope. Organ-culture of the rudiments was performed in 10 $cm^2$ plastic culture dishes. A Teflon carrier with a nylon mesh (20 $\mu$m pore size) was mounted in the dish, keeping the explants floating and ensuring optimal gas exchange and nutritional conditions. The right and the left femora from each animal were randomly assigned to the experimental or control group. The control groups were kept in 3 ml B with 50 $\mu$g/ml ascorbate. In the experimental group the growth medium was supplemented with either 10 mM Cr, 20 mM Cr, 1 mM GPA, 5 mM GPA, or 10 mM GPA. The growth medium was renewed every second day until day 10. Culture was carried out at 37,5 °C and in a 5 % $CO_2$ atmosphere. At 10 days the wet weight of each femora was determined on a microbalance. The result of each experimental

femora was expressed relative to its collateral control. For the histological evaluation, the femora were fixed in 4% formaldehyde, dehydrated and embedded in methylmethacrylate. The 6 μm sections were stained by Pentachrome-Movat.

**Culture condition**

All the cultures were kept at 37°C in a humidified atmosphere of 5 % $CO_2$, 95 % air.

All culture media were supplemented with 50 μg/ml ascorbate. To analyze the collagen types, 60 μg/ml beta-aminopropionitrile (beta-APN) was added to the culture medium. During cell isolation and inoculation no ascorbate was used to increase plating efficiency. No antibiotics and no beta-glycerophosphate were added. The media were completely changed every 48 hours (60 mm culture dish 5 ml ; 35 mm culture dish 2 ml).

**Morphology**

Alkaline phosphatase activity (histochemically)

The cells were histochemically stained for the alkaline phosphatase as described in the Sigma technical Bulletin no. 85L.

Principle

Gently fixed cells were incubated in a solution containing naphtol AS-MX. As a result of phosphatase activity, naphtol AS-MX was liberated and immediately coupled with a diazonium salt forming an insoluble, blue pigment at sites of phosphatase activity.

Solutions

- Fixative

  2 vol. Citrate buffer; dilute citrate concentrate 1:50

  3 vol. acetone

- Stain

  dissolve content of 1 capsule Fast Blue in 48 ml distilled water on a magnetic stirrer.

  add 2 ml of naphtol AS-MX solution just before use.

Procedure

1. wash 3 x in TBSS
2. fixation 5 min. at 20°C
3. wash 3x with distilled water
4. stain 30' in the dark at room temperature (RT)
5. wash 3x with distilled water.

**Mineralization**

Principle

The most specific method for detecting calcified matrices is the von Kossa reaction. Silver staining indicates the presence of calcium phosphate aggregated with certain organic acids. Structural detail are completely obscured by the dark precipitate. Calcified tissue components are darkened in various shades from light brown to deep black, irrespective of their mineral content.

Solution

- Silver nitrate

  5 % $AgNO_3$ in distilled water

- Pyrogallol

  1 % in distilled water

- Sodium thiosulphate

  1 % $Na_2S_2O_3$ 5 $H_2O$ in distilled water

Procedure

| | |
|---|---|
| 1. fixation in 4 % formaldehyde | 30 min. |
| 2. wash in distilled water | 3x |
| 3. silver nitrate | 30 min. in the dark |
| 4. wash with distilled water | 5x |
| 5. pyrogallol | 5 min. |
| 6. wash with distilled water | 5x |
| 7. sodium thiosulphate | 10 min. |
| 8. wash with distilled water | 5x |

**TEM preparation**

Principle

In an electron microscope the specimen is exposed to very high vacuum. Therefore, the tissue has to be fixed and stained with heavy metals to give contrast and only very dense material deflects electrons and forms images. The tissue is impregnated with heavy metals (uranium, lead) either before or/and after

sectioning. Because electrons do not penetrate very deeply into the tissue, very thin sections (50-100 nm) have to be cut with either a glass or a diamond knife on an ultra microtome. For ultrathin sectioning the specimen has to be dehydrated and penetrated with monomeric resin which polymerizes.

For chemical fixation glutaraldehyde is mostly used. It cross-links the proteins covalently to their neighbours. In order to stabilize the lipids, especially the cell membranes, osmium-tetroxide is used as a postfixation. To enhance the contrast the tissue is treated *en block* with uranyl acetate and the sections are subsequently stained with uranylacetate and lead citrate.

Solutions

- 0,2 M Cacodylate buffer pH 7,4

  Stock A 25 ml

  Stock B 1,35 ml

  distilled water ad 100 ml

  Stock A 10,7 g Cacodylic acid sodium salt Trihydrate

  250 ml Distilled water

  Stock B 0,2 M HCl

- Fixation

  25 % glutaraldehyde (EM grade) 2 ml

  0,2 M cacodylate buffer pH 7,4 10 ml

  distilled water ad 20 ml

- Postfixation 1 % $OsO_4$ in 0.1 M cacodylate buffer pH 7,4

  1 vol. 2 % $OsO_4$

  1 vol. 0,2 M Cacodylate buffer pH 7,4

  2 % $OsO_4$

fracture glass vial

add distilled water

sonicate 5 min.

filter through 0,45 mm filter (Millex)

keep in dark at 4°C

- 2 % aqueous uranyl acetate

Procedure

| | |
|---|---|
| 1. Fixation at 20°C | 20 min. |
| 2. rinse in 0,1 M cacodylate buffer pH 7,4 | 3x 30 s |
| 3. post-fixation at 4°C | 1 h |
| 4. rinse in distilled water | 3x 30 s |
| 5. uranyl acetate at room temperature. | 1 h |
| 6. dehydration in a graded series of ethanol: | |
| 7. 70 %, 80 %, 90 %, 100 %, 100 %, 100 %. | every 5 min. |
| 8. LR White (Polysciences). | > 2 h |
| 9. Polymerization at 60°C. | overnight |

Ultrathin sections

Principle

Ultrathin sections were cut either with a glass knife or with a Drukker Diamond knife on a LKB III Microtome, placed on Formvar coated copper grids, and stained with heavy metals.

Solutions

- 5 % uranyl acetate

  1 g/20 ml.

- lead citrate according to Reynolds

| | |
|---|---|
| $Pb(NO_3)_2$ | 0,67 g |
| Sodium citrate | 0,88 g tri sodium citrate dihydrate |
| | 15 ml distilled water |

gentle shaking for 15 min.

add 4 ml 1 N NaOH, white precipitate dissolves

fill up to 25 ml distilled water

add distilled water to 25 ml

Filter both solutions through a Whatman No. 50 (hardened) before use.

Procedure

All solutions were placed as drops on a parafilm. Individual grids were placed onto the droplets, to floating, section side down. Solid NaOH pellets were placed in a plastic dish in the same chamber to absorb $CO_2$ from the air to prevent carbon dioxide precipitation of lead salts. Both the staining solutions and the solid NaOH pellets were covered with a lid.

| | |
|---|---|
| 1. distilled water | |
| 2. 5 % uranyl acetate | 10 min. |
| 3. distilled water | 2x |
| 4. lead citrate | 10 min. |
| 5. M NaOH | 3x 30 s |
| 6. distilled water | 2x |

7. remove the remaining small amounts of water between the prongs of the forceps with filter paper and dry the grids on Whatman No. 50 filter paper with the section side up. When the grids were dry, they were placed in the storage box ready for use.

The sections were examined on a JEOL JEM 100 CX transmission electron microscope operated at 100kV. Micrographs were taken on Kodak EM 4303 film at standard magnifications of 2000, 5000, 20000 or 33000 times. Pictures were printed onto multigrade paper.

**Cell viability (MTT)**

The Böhringer 'Cell Proliferation Kit I (MTT)' was used for the assay, but we used a different solvent to dissolve the MTT crystals.

Principle

Originally, Mosmann, 1983 described the general principle involved in the detection of cell growth/cell death as indicated by the conversion of the tetrazolium salt (MTT) to the coloured formazan by mitochondrial dehydrogenases. The concentration of this can then be measured spectrophotometrically.

Procedure

1. MTT Stock (5 mg/ml in sterile PBS) from Böhringer was diluted 110 with complete growth medium and sterile filtering.
2. The cells were incubated in 2 ml/10cm$^2$ MTT solution at 37°C for 3h.
3. The supernatant was carefully removed.
4. 4 ml/10cm$^2$ dimethylsulphoxide (DMSO) was added.

5. The dishes were placed on a shaker until the crystals were completely dissolved.

6. The absorbance of the supernatant (3 aliquots/dish) was read at 550 nm versus DMSO.

Comments

If the absorbance was higher than 1, the samples were diluted with DMSO.

**Cell viability (neutral red, NR)**

The method as described in (Lindl et al. 1994) was used.

Principle

The uptake of NR into lysosomes is independent of the metabolic status of the cell.

Solutions

- 0,5 mg Neutral red /ml growth medium, warmed up to 37°C for at least 2h, sterile filtering
- Extraction buffer
  50% ethanol in 1 % acetic acid

Procedure

1. The cells were incubated in 2 ml/10cm$^2$ NR solution at 37°C for 3h.
2. The supernatant was removed,

3. washed with PBS, at least 3 times, until no crystals were present.
4. Addition of 4 ml/10cm$^2$ extraction buffer.
5. The absorbance of the supernatant (3 aliquots/dish) was read at 540 nm versus extraction buffer.
6. If the absorbance was higher than 1, the samples were diluted with extraction buffer.

**Statistics**

The mean value and the standard deviation consisted of n independent experiments. The values for the individual experiments were gained from the mean of 3 aliquots of the same dish. To compare the treatment contrasts analysis of variance models were evaluated.

In experiments carried out as paired designs a model accounting for the animals considered as blocks were examined. Main effects and interaction effects were examined by F-Tests.

'Least Squares Means' were calculated to yield average means accounted for the other variables in the model. LS Means were compared by using Tukey's multiple range test.
QQ-Plots of the residuals and Tukey-Anscombe plots (residuals x predicted) were analyzed to check for normal distribution assumption.

Results

Monolayer Cell culture

Cell viability and metabolic activity

With respect to cell viability, in all groups, neutral red (NR) stained mainly the cells at the edge and the top of the nodules as well as the cells between them. Staining with trypan blue showed that the cells/matrix between the nodules and the nodules themselves were stained.

Preliminary quantitative data on the NR uptake showed that the Cr and the GPA groups had similar results as the control group at 2 weeks. Concerning the metabolic activity measured by the MTT reaction, the Cr groups were slightly stimulated when compared to the control, but the 5 M or the 10 mM GPA had lower values than the control, indicating some inhibition of the GPA at these particular concentration. The 1 mM GPA group was similar to the control. At 3 weeks, all experimental groups had a lower NR uptake than the control. The Cr stimulated the MTT reaction, and the 1 mM or 5 mM GPA had lower values than the control. The 10 mM GPA was comparable to the control. These results were indicating that the Cr had a stimulatory effect on the metabolism of the cells and the GPA had some inhibition on the mitochondrial activity of the cells.

In the further experiments to quantify the viability and the metabolic activity of the cells, the creatine groups were only used. Statistical analysis of the NR uptake (Fig. 1) showed that

there was a small but significant interaction effect ($p<0.05$). This meant that the effect of treatment with creatine was not similar at the different time points. The NR uptake of the control group at 1 week was significantly lower than that of 2 and 3 week ($p<0,03$, respectively $p<0,0002$). The NR uptake of the 10 mM Cr group was significantly higher at 3 weeks as compared to that at 1 week ($p<0,02$). At 1 and 2 weeks there was no significant difference between the groups. At 3 weeks the control group was significantly ($p<0,008$) higher than the 20 mM Cr group. The increase in the NR uptake of the control group during the culture indicated that there was an increase in the cell number. The difference of the control group and the 10 mM Cr was no-significant. This showed that there was no toxic effect of the creatine at this particular concentration. This was in contrast to the 20 mM Cr, which had an significantly lowered NR uptake when compared to the control group. This indicated some toxic effects on the proliferation of the cells.

Concerning the metabolic activity (MTT) of the cells (Fig. 2), creatine had an effect on osteoblasts in culture. At 1 week all groups were similar. At 2 weeks the control group was significantly lower than the 10 mM Cr and the 20 mM Cr ($p<0,015$, respectively $p<0,0025$). At 3 weeks both the 10 mM Cr and 20 mM Cr were significantly higher than the control group ($p<0,001$). These data showed that in general creatine stimulated the metabolic activity of osteoblasts from the second week onwards.

Morphology

After 1 week the cells in all groups formed a monolayer with ALP positive cells. Some cells had a really high ALP activity. After 2 weeks all groups formed some small mineralized nodules. After 3 weeks the overall staining for ALP activity was similar in all groups. At higher magnification the GPA groups showed a different staining pattern for the ALP activity compared with the control and the Cr groups. The cell density around the nodules was lower than in the control and the Cr groups. At 3 weeks the mineralized nodules increased in size and number compared with 2 weeks. All the experimental groups showed a higher mineralization than the control group. The calcification pattern of the GPA groups was different from the control and the Cr groups in such that the mineralization was not limited to the nodules and more single cells showed calcification than the control and Cr groups.

In the further experiments to quantify the calcification by image analysis of a center area (123 $mm^2$) of the culture dish, only the creatine groups were compared to the control groups, with the GPA treated cells not further evaluated. Statistical analysis showed that the calcified area in the 20 mM Cr group (Fig. 3) was significantly higher than the one in the control group ($p<0,02$) at 2 weeks. At 3 weeks, 10 mM Cr group had more mineralization than the control, whereas the 20 mM Cr was less effective, but there was no significant difference between the various groups.

TEM-Monolayer

The ultrastructure of the control group at 1 % FCS was similar to the cells kept at 10 % FCS.

The ultrastructure showed that there were no obvious differences between the control, the 10 mM Cr group, the 20 mM Cr group the 1 mM GPA group, and the 5 mM GPA group.

In all groups there was collagen production and mineralization. The cytoplasm of cells had the typical features of osteoblasts such as a well developed rER, Golgi area, mitochondria, vesicles, micro filaments. The cells had many cell processes which were in close contact to each other. There was abundant collagen production. The collagen fibrils were seen in membrane folds. The diameter of the fibrils was rather uniform. In the area of mineralization, the individual fibrils seem to coalesce into larger units. The mineralization pattern was similar in all groups. There were high density needle-like structures at the lowest cell layers. At the mineralization front the same material was observed around collagen fibrils and in close apposition to the plasma membranes. Mineralized patches were seen in the collagenous matrix. In areas with high calcification the details of the matrix were no longer visible.

Micromass cell culture

The NR uptake was similar in all groups at 1 and 2 weeks. At 3 weeks the 20 mM Cr groups had a significantly lower NR uptake ($p<0,005$, respectively $p<0,003$) than the control and the 10 mM Cr group.

The mitochondrial activity (MTT conversion) was similar in all groups at 1,2, and 3 weeks. However, the Cr groups at 10 mM and 20 mM concentration had a significantly higher MTT reaction at 2 weeks than at 1 week ($p<0,02$, respectively $p< 0,006$). At 3 weeks, the 20 mM Cr had a significantly lower MTT conversion than at 2 weeks ($p<0,015$).

Concerning the mineralized area (Fig. 4), the creatine groups at 10 mM and 20 mM concentrations had significantly more mineralization ($p<0,00025$) than the control at 2 weeks. At 3 weeks the mineralized area was significantly higher in the creatine groups at 10 mM and 20 mM concentrations than in the control ($p<0,0035$, respectively $p<0,03$). Furthermore, the control and the 10 mM Cr groups showed a significantly higher mineralization at 3 weeks than at 2 weeks ($p<0,0005$, respectively $p<0,0015$).

Organ Culture

Femora

The control (Fig. 5) had significantly lower wet weights than 10 mM Cr ($p<0,005$), 20 mM Cr ($p<0,001$), 5 mM GPA ($p<0,0005$) and 10 mM GPA ($p<0,015$). The results of 1 mM GPA were not significantly different from the control.

Assessment of the experimental data

There was a small but significant interaction effect of creatine in the NR uptake in monolayer cultures. This meant that the effect of the treatment with creatine was not similar at the

different time points. In the control group there was a significant increase in the NR uptake during the culture. This was due to an increase in the cell number. At 1 and 2 weeks there was no significant difference between the groups. However, the effect of the 10 mM Cr on the NR uptake was significant at 3 weeks compared to that at 1 week. At 3 weeks the 20 mM Cr group was significantly lower than the control group. This indicated some toxic effects, which resulted in a reduced proliferation of the cells. This was not observed in the 10 mM Cr group, which was similar to the control group. This showed that there was no toxic effect of the creatine at this particular concentration of 10 mM. In the micromass cultures, the NR uptake was similar in all groups at 1 and 2 weeks. However at 3 weeks the 20 mM Cr had significantly less than the control and the 10 mM Cr. In contrast to the monolayer cultures, the NR uptake was not reduced during culture. This could be explained by the fact that in micromass cultures, the cells were migrating off the initially inoculated drop of cells and so the cell number is slowly increasing.

The results concerning the metabolic activity of monolayer cultures osteoblasts showed a significant stimulation of these cells by creatine at both concentrations, 10 mM and 20 mM, from the second week onwards. In the micromass cultures, the increase in the MTT conversion was only significant in the Cr groups at 2 weeks compared to the one 1 week. This indicated that the micromass culture behave differently than the monolayer cultures. This was not astonishing because in the micromass cultures, the cells have a very early cell-cell contact and so the differentiation process started earlier than in the monolayer

cultures where the cells have first to proliferate to make cell-cell contacts. Nevertheless, the Cr significantly stimulated the metabolic activity of the micromass cultures at the early mineralization at 2 weeks when compared at 1 week.

In all groups, NR stained mainly the cells at the edge and the top of the nodules and between them. Staining with trypan blue in all groups showed that the cells at the bottom of the culture dish stained as well as those in the nodules. This could either be attributed to an artefact of staining or it might be that the cell membrane of the stained cells was really damaged. Concerning the artefact possibility, trypan blue would also stain extracellular proteins. An indication of the presence of damaged cell membranes was obtained from the TEM ultrastructure studies of monolayer cultures. Some of the cells near the culture dish surface had electron dense, needle-like material in the cytoplasm. It could be that the lower cells of the mineralizing nodule did not get enough nutrition or oxygen by diffusion through all other cell layers. It is very important that the cells stay alive because only viable cells can regulate mineral deposition and prevent dystrophic calcification. The presence of dead cells can lead to an increased mineralization.

After 2 weeks all groups formed some small mineralized nodules which increased in size and number after 3 weeks. Calcification was also observed in single cells. Mean values were higher in the Cr groups than the control after 2 and 3 weeks. In the micromass cultures, the Cr groups had significantly more mineralization than the controls.

Thus, Cr enhanced the formation of mineralized nodules by increasing the metabolic activity of the osteoblasts in cultures. It is suggested that there is an elevation in PCr turnover during tissue mineralization because the creatine phosphate concentration in calcified cartilage is low and the activity of the kinase in this zone is high. Furthermore, the energy metabolism in cartilage may affect the morphogenic events of skeletal growth.

There is evidence that mineralizing cells require a large amount of energy. Differentiating osteogenic cells have mitochondria with condensed cristae that represent an increased rate of energy metabolism. These mitochondria are particularly abundant in the differentiation stage and decline as the culture matures. Mineralization is thought to be associated with an optimal level of energy metabolism rather than extreme hypo- or hyperoxia.

Increased glycolysis with constant mitochondrial activity results in an augmented energy metabolism and increased ATP production. This increased availability of ATP could be a reason why osteoblasts synthesize more collagen when they are exposed to a high pH. An increased cell differentiation, during the formation of bone and cartilage, is accompanied by enhanced activities of ATPase and lactate, malate and glucose-6-phosphate dehydrogenases. Maximum activity is observed at the onset of the matrix deposition, followed by a decrease of enzyme activities during the transformation of osteoblasts to mature osteocytes and at the hypertrophy of chondrocytes. Histochemical ATPase activity, detected in osteoblasts, parallels the metabolic activity and viability of these cells. The ATPase activity in

bone and cartilage cells is far less than in skeletal muscle, blood vessels and bone marrow. Osteoclasts reveal strong ATPase activity followed in intensity by osteoblasts, osteochondrogenic cells and lastly osteocytes. Cartilage cell activity, determined in this way, is generally weaker than osteoblastic activity. Young cell compartments reveal greater activity than those of older animals, with peak activity usually observed to 5 weeks of age. With increasing age and reduced functional demands the ATPase activity diminishes except in articular cartilage cells.

Inhibition of the glycolysis causes both a reduction in collagen synthesis and a hypermineralization in tibiae of chick embryos over a wide range of [Ca x Pi] in the medium (Pi 0,5 mM - 3,0 mM and 1,8 mM $Ca^{2+}$). Furthermore, in the absence of glutamine there is more cell necrosis. Glutamine enters the citric acid cycle at a-ketoglutarate and provides biosynthetic precursors and NADH. NADH enters the oxidative phosphorylation and provides ATP. Inhibition of the activity of NAD-dependent enzymes associated with the production of ATP impairs cartilage formation resulting in limb shortening.

GPA, a competitive inhibitor of Cr entry into cells, seems to have adverse effects on both the metabolism and the viability of the cells, but mineralization is increased. This could be explained by the fact that cell death can also lead to mineralization. Since metabolic activity of creatine treated cells was generally higher compared to controls, the same parameter was lower in GPA, it was concluded that increased mineralization in the Cr treated groups was due to the metabolic stimulation of osteoblasts, whereas the one in GPA treated cells

was mainly due to cell death. It is shown that growth plate cartilage cannot adapt to the metabolic stress imposed by GPA administration resulting in a disturbed enchondral bone formation in vivo and in vitro. The zone of calcified cartilage is wider and extends into the metaphysis. The hypertrophic chondrocytes are vacuolated and poorly columnated and mineralization is less abundant and occurs also in the transverse septa. Vascular invasion of the tissue is poor. There is a reduction in the osteoid formation. GPA interferes with the synthesis of pro-a type II and type X collagen in cultured chondrocytes. In long-term shell-less culture in the presence of GPA, the total CK activity is not altered but the CK isoenzyme profile is disturbed. The activity of BB-CK is suppressed in the long bones, but the isoenzyme distribution of calvariae is not affected. Normal embryonic cartilage contains nearly equal proportions of MM-CK and BB-CK. Embryonic calvariae and bone mainly express BB-CK. Feeding of rat and mice with GPA progressively decreases the concentrations of Cr and PCr in heart and skeletal muscle and leads to marked morphological changes mainly affecting mitochondria. A population of enlarged, rod-shaped mitochondria with characteristic crystalline intramitochondrial inclusions appears in adult rat cardiomyocytes in vitro. This phenomenon is fully reversible if the cell culture medium is supplemented with Cr. The appearance of highly ordered intra-mitochondrial inclusions correlates with a low intracellular total Cr content. Immunofluorescence and immuno-electron microscopy shows that these inclusions are enriched for Mi-CK. In the GPA treated osteoblasts, the mitochondria were similar to the control and Cr

groups. So osteoblasts respond differently to GPA than do muscle cells. It is shown that GPA had comparably less influence on the Cr and PCr contents of brain. Soleus mitochondria show a 4 fold increase in Mi-CK protein and a 3 fold increase in adenine nucleotide translocator protein compared to the control.

**Conclusions**

Creatine stimulates via the action of creatine kinase and other enzymes regulated by creatine or phosphocreatine, like AMP-dependent protein kinase the mineralization of osteoblasts in culture by increasing the metabolic activity of the cells in monolayer culture. In micromass cultures the creatine enhanced the mineralization but the metabolic activity was similar to the control. However, at 2 weeks the MTT conversion was significantly increased in the creatine group when compared to 1 week. Creatine had probably some effects on the differentiation process of the cells in this cell culture model. During nodule formation and subsequent calcification, the cells need a large amount of chemical energy. Biosynthesis of matrix collagen and proteo-glycans, and/or the proliferation of the cells are increased. Creatine, as an external energy supply, has the advantage that it does not decrease the pH in the growth medium, thus avoids an inhibition of glycolysis and collagen synthesis.

Creatine also increases the wet weight of embryonic femora (Fig. 5) in organ culture. Indicating that not only bone but also cartilage cells benefit from external creatine supply. The biosynthesis of the matrix collagen and proteoglycan, and/or the proliferation of the cells are stimulated.

Creatine can therefore be applied as a food additive or supplement for humans and animals to support the recovery after trauma and orthopaedic surgery of fractures and bone defects.

Creatine has also potential to stimulate the metabolism of osteoblasts in patients suffering from osteoporosis.
Also the treatment of degenerative cartilage diseases, such as arthritis is supported by creatine.

The treatment of large bone defects is still a demanding task for surgeons. Patients suffering from large bone defects can be treated with bone grafting from the illiac crest to the defect or by applying callus distraction or segment transport. All these procedures are very painful for the patient and additionally the amount of bone graft is limited. The use of tissue engineering offers a solution to this problem. Bone forming cells (osteoblasts, mesenchymal stem cells, periosteal cells, stromal bone marrow cells or satellite cells of the muscle) as well as chondroblasts of healthy individuals or from a patient himself are cultured as monolayers, micromass cultures or in a three-dimensional, biodegradable scaffold in the presence of creatine. At a later point in time, the bone or cartilage cells

or cell-seeded sponges, foams or membranes will be transferred to the defect in the patient. The most critical step in this approach is the cell culture work. It is fundamental that the cells survive, proliferate and differentiate *in vitro*. Therefore, culture conditions need to be optimal. In this respect, addition of creatine to the culture medium as a supplement is beneficial.

Although bone and cartilage cells express creatine kinase, albeit at relatively low levels compared to muscle and brain cells, it is surprising that over- expression of creatine kinase together with creatine supplementation improved proliferation, metabolic stability and resistance towards different stressors, e.g. toxins, heat, metabolic overload etc. of cartilage and bone cells. Thus bone forming cells (osteoblasts, periostal cells, stromal bone marrow cells or satellite cells of muscle) and cartilage forming cells (chondroblasts) removed from healthy individuals or from a patient to be treated are brought into cell culture and transfected with complementary DNA coding for creatine kinase isoforms (either cytosolic muscle-type MM-CK, cytosolic ubiquitous brain-type BB-CK or the heterodimeric MB-CK hybride enzyme, or sarcomeric- or ubiquitous mitochondrial Mi-CK's or combinations thereof). Complementary DNA (cDNA) can be obtained by reverse transcribing (RT) mRNA of CKisoenzymes, by RT-polymerase-chain reaction (RT-PCR), or by other methods using the appropriate primers corresponding to the respective CKisoenzymes.

The methods of gene transfer for cDNA's encoding for creatine kinase isoforms will encompass the entire selection of possible transfection techniques, as well as new techniques developed and made accessible to the public domain in the future, such as transfection via microinjection of cells, microsphere bombardment or DNA-precipitate transfection, as well as transfection via various viruses, viral and non-viral vectors or plasmids (single copy- and multi-copy plasmids), cosmids or artificial chromosomes. Creatine kinase expression is made under the control of weak or strong tissue specific promotors. Built-in selection markers, e.g. resistance towards antibiotics, toxins or others, make it possible to select for transfected cells which then are expanded in culture as described above in the presence of 1 to 20 mM creatine.

Cartilage or bone cells transfected with creatine kinase cDNA, made to overexpress creatine kinase isoenzyme(s) are then selected on a selection medium and expanded and cultivated either as monolayers, micromass cultures or on three-dimensional, biodegradable scaffolds or tissue sponges (as described above) to form in vitro genetically engineered cartilage- and bone pre-tissues which can be transplanted into the areas of cartilage and/or bone defects. For example, such transfected cartilage cells can be injected into arthritic joints to repopulate the areas of defect and repair chondro-degenerative defects in this joint by proliferation and producing new chondrocyte-derived extracellular

matrix. Similarly, transfected bone-forming cells can be reimplanted into areas of bone defect to initiate regeneration and growth of bone mass in patients.

Since creatine kinase and creatine/phosphocreatine play an important role in the generation and maintenance of cartilage- and bone tissues, such tissues, genetically engineered to overexpress creatine kinases and being supplemented by externally added creatine or creatine analogues, are growing better after transplantation into areas of cartilage and/or bone defect in patients supplemented orally and/or locally with creatine.
Genetic engineering of creatine kinase into cartilage and bone cells in conjunction with creatine supplementation improves the proliferation, growth and specific function of these cells, e.g. the formation of extracellular cartilage- or bone-specific matrix. This metabolic engineering procedure followed by creatine supplementation is beneficial for cartilage and bone formation, healing and repair, as well as for mineralization.

The concentration of the creatine compound in the culture medium should preferably be in the range of 10 - 20 mM. The culture medium typically contains 0,1 % to 5,0 %, preferably 0,5 % to 2 % foetal calf serum. Furthermore the culture medium should contain 10 to 250 µg, preferably 25 to 100 µg ascorbic acid or an equivalent amount of a pharmaceutically acceptable ascorbate. The cell culture is started with 2'000 to 100'000 cells, preferably 10'000 to 50'000 cells.

In a preferred embodiment of the invention the creatine compound is administered in combination with hormones, preferably selected from the group of parathyroid hormone-related protein, thyroid hormone, insulin, sex steroids (estrogen, androgen, testosterone), prostaglandins, or glucocorticoids.

In further preferred embodiment the creatine compound is administered in combination with intermittent administration of parathyroid hormone, preferably in combination with $1,25(OH)_2$ vitamin $D_3$ and analogues or metabolites of vitamine D, calcitonine, estrogen, or bisphosphonates.

A further preferred embodiment includes administration of the creatine compound in combination with vitamins, preferably selected from the group of $1,25(OH)_2$ vitamin $D_3$ and analogues or metabolites of vitamine D, of vitamin C/ascorbate or of retinoids.

A further preferred embodiment includes administration of the creatine compound in combination with growth factors, preferably selected from the group of insulin like growth factors (IGF), transforming growth factor b family (TGF-b), bone morphogenic proteins (BMP), basic fibroblastic growth factor (bFGF), platelet derived growth factor (PDGF), or epidermal growth factor (EGF).

A further preferred embodiment includes administration of the creatine compound in combination with cytokines, preferably selected from the group of interleukins (IL), interferons, or leukaemia inhibitory factor (LIF).

A further preferred embodiment includes administration of the creatine compound in combination with matrix proteins, preferably selected from the group of collagens, glycoproteins, hyaluronan, or proteoglycans.

As glycoproteins those selected form the group of:

a) alkaline phosphatase,

b) osteonectin (ON),

c) gamma-carboxy glutamic acid-containing proteins, preferably matrix gla protein or osteocalcin or bone gla protein (OC),

d) arginine-glycine-asparagine-containing proteins, preferably thromspondin, fibronectin, vitronectin, fibrillin, osteoadherin, sialoproteins (osteopontin or bone sialoprotein BSP)

have shown a particular usefulness.

As proteoglycans those selected form the group of:

a) aggrecan,

b) versican,

c) biglycan,

d) decorin

have shown a particular usefulness.

In a further preferred embodiment the creatine compound is administered in combination with serum proteins, preferably selected from the group of albumin or alpha-2HS glycoprotein.

A further preferred embodiment includes administration of the creatine compound in combination with enzymes, preferably selected from the group of metalloproteinases, collagenases, gelatinases, stromelysins, plasminogen activators, cysteine proteinases or aspartic proteinases.

A further preferred embodiment includes administration of the creatine compound in combination with calcium salts, bone meal or hydroxyapatite.

A further preferred embodiment includes administration of the creatine compound in combination with fluoride salts, preferably sodium fluoride or monosodium fluorophosphate.

A further preferred embodiment includes administration of the creatine compound in combination with peptides, preferably selected from the group of amylin, vasoactive agents or neuropeptides.

A further preferred embodiment includes administration of the creatine compound in combination with antioxidants, preferably selected from the group of cysteine, N-acetyl-cysteine, glutathions or vitamins A, C, D or E.

A further preferred embodiment includes administration of the creatine compound in combination with a substance selected from transferrin, selenium, boron, silicon, or nitric oxide.

In a preferred embodiment of the invention the agent is essentially free of dihydrotriazine. It has been found that dihydrotriazine is a toxic impurity of commercially available creatine and that it has an adverse effect for the patient.
For the same reason the agent should be essentially free of dicyano-diamide which is also toxic impurity of commercially available creatine.
It is further advantageous to an agent which is essentially free of creatinine as a natural degradation product of creatine.

The agent according to the invention is administered to a human patient preferably in an amount of 1,4 to 285 mg per day.

The various modifications and preferred embodiments characterized in the dependent claims have produced a stimulatory effect on bone and/or cartilage.

While the foregoing description and drawings represent the preferred embodiments of the present invention, it will be obvious for those skilled in the art that various changes and modifications may be made therein without departing from the true spirit and scope of the present invention.

Claims

1. Use of creatine compounds including analogues or pharmaceutically acceptable salts thereof, for the preparation of an agent for the treatment of bone or cartilage cells and tissues.

2. Use according to claim 1, characterized in that said agent is used for the therapeutic treatment of bone or cartilage diseases, preferably of osteoporosis, osteoarthritis or periodontitis, or for the prophylactic treatment of said bone or cartilage diseases.

3. Use according to claim 1, characterized in that said agent is used for promoting growth and mineralisation of bone or cartilage cells and tissues.

4. Use according to claim 1, characterized in that said agent is used for conservative or operative treatments of bone fractures or bone defects.

5. Use according to claim 4, characterized in that said agent is incorporated in a bone or cartilage graft to be applied to said fractures or defects.

6. Use according to claim 4, characterized in said agent is incorporated in three dimensional constructs of osteoblasts, chondrocytes or mesenchymal stem cells designed for tissue engineering of said bone or cartilage defects.

7. Use according to claim 1, characterized in that, bone or cartilage forming cells obtained from a healthy individual or a particular patient are cultured in the presence of said agent to form a three-dimensional cell assembly which is transferrable in a subsequent step to a specific location having a bone or cartilage defect of the same particular patient.

8. Use according to claim 1, characterized in that said agent is used for improving acceptance and osseous integration of bone implants.

9. Use according to claim 8, characterized in that said bone implants are endoprosthesis, preferably joint endoprosthesis.

10. Use according to one of the claims 1 to 9, characterized in that said creatine compound is selected from the group of creatine, creatine phosphate, creatine pyruvate, cyclocreatine, homocreatine or homocyclocreatine.

11. Use according to one of the claims 1 to 10, characterized in that said creatine compound is administered in combination with hormones, preferably selected from the group of:

a) parathyroid hormone-related protein,

b) thyroid hormone,

c) insulin,

d) sex steroids (estrogen, androgen, testosterone),

e) prostaglandins,

f) glucocorticoids.

12. Use according to claim 11, characterized in that said creatine compound is administered in combination with intermittent administration of parathyroid hormone, preferably in combination with $1,25(OH)_2$ vitamin $D_3$ and analogues or metabolites of vitamine D, calcitonine, estrogen, or bisphosphonates.

13. Use according to one of the claims 1 to 12, characterized in that said creatine compound is administered in combination with vitamins, preferably selected from the group of:

a) $1,25(OH)_2$ vitamin $D_3$ and analogues or metabolites of vitamine D,

b) vitamin C/ascorbate,

c) retinoids.

14. Use according to one of the claims 1 to 13, characterized in that said creatine compound is administered in combination with growth factors, preferably selected from the group of:

a) insulin like growth factors (IGF),

b) transforming growth factor b family (TGF-b),

c) bone morphogenic proteins (BMP),

d) basic fibroblastic growth factor (bFGF),

e) platelet derived growth factor (PDGF),

f) epidermal growth factor (EGF).

15. Use according to one of the claims 1 to 14, characterized in that said creatine compound is administered in combination with cytokines, preferably selected from the group of:

a) interleukins (IL),

b) interferons,

c) leukaemia inhibitory factor (LIF).

16. Use according to one of the claims 1 to 15, characterized in that said creatine compound is administered in combination with matrix proteins, preferably selected from the group of:

A) collagens,

B) glycoproteins,

C) hyaluronan,

D) proteoglycans.

17. Use according to claim 16, characterized in that said glycoproteins are selected form the group of:

a) alkaline phosphatase,

b) osteonectin (ON),

c) gamma-carboxy glutamic acid-containing proteins, preferably matrix gla protein or osteocalcin or bone gla protein (OC),

d) arginine-glycine-asparagine-containing proteins, preferably thromspondin, fibronectin, vitronectin, fibrillin, osteoadherin, sialoproteins (osteopontin or bone sialoprotein BSP).

18. Use according to claim 16, characterized in that said proteoglycans are selected form the group of:

a) aggrecan,

b) versican,

c) biglycan,

d) decorin.

19. Use according to one of the claims 1 to 18, characterized in that said creatine compound is administered in combination with serum proteins, preferably selected from the group of:

a) albumin

b) alpha-2HS glycoprotein

20. Use according to one of the claims 1 to 19, characterized in that said creatine compound is administered in combination with enzymes, preferably selected from the group of:

a) metalloproteinases,

b) collagenases,

c) gelatinases,

d) stromelysins,

e) plasminogen activators,

f) cysteine proteinases,

g) aspartic proteinases.

21. Use according to one of the claims 1 to 20, characterized in that said creatine compound is administered in combination with calcium salts, bone meal or hydroxyapatite.

22. Use according to one of the claims 1 to 21, characterized in that said creatine compound is administered in combination with fluoride salts, preferably sodium fluoride or monosodium fluorophosphate.

23. Use according to one of the claims 1 to 22, characterized in that said creatine compound is administered in combination with peptides, preferably selected from the group of:

a) amylin,

b) vasoactive agents,

c) neuropeptides.

24. Use according to one of the claims 1 to 23, characterized in that said creatine compound is administered in combination with antioxidants, preferably selected from the group of:

a) cysteine,

b) N-acetyl-cysteine,

c) glutathions,

d) vitamins A, C, D or E.

25. Use according to one of the claims 1 to 24, characterized in that said creatine compound is administered in combination with a substance selected from:

a) transferrin,

b) selenium,

c) boron,

d) silicon,

e) nitric oxide.

26. Use according to one of the claims 1 to 25, characterized in that said bone cells are selected form the group of osteoblasts, periosteal cells, stromal bone marrow cells, satellite cells of the muscle tissue and mesenchymal stem cells.

27. Use according to one of the claims 1 to 25, characterized in that said cartilage cells are selected form the group of chondroblasts, chondroclasts and mesenchymal stem cells.

28. Use according to one of the claims 6 to 27 , characterized in that said bone or cartilage cells are cultured as monolayers, micromass culture or in a three-dimensional, biodegradable scaffold.

29. Use according to one of the claims 7 to 27 , characterized in that said three-dimensional cell assembly has the structure of a seeded sponge, foam or membrane.

30. Use according to one of the claims 7 to 29 , characterized in that the concentration of said creatine compound in the culture medium is in the range of 10 - 20 mM.

31. Use according to one of the claims 7 to 30, characterized in that the culture medium contains 0,1 % to 5,0 %, preferably 0,5 % to 2 % foetal calf serum.

32. Use according to one of the claims 7 to 31 , characterized in that the culture medium contains 10 to 250 µg, preferably 25 to 100 µg ascorbic acid or an equivalent amount of a pharmaceutically acceptable ascorbate.

33. Use according to one of the claims 7 to 32 , characterized in that said cell culture is started with 2'000 to 100'000 cells, preferably 10'000 to 50'000 cells.

34. Use according to one of the claims 1 to 33 , characterized in said agent is essentially free of dihydrotriazine.

35. Use according to one of the claims 1 to 34 , characterized in said agent is essentially free of dicyano-diamide.

36. Use according to one of the claims 1 to 35 , characterized in said agent is essentially free of creatinine as a natural degradation product of creatine.

37. Use according to one of the claims 1 to 6 or 9 to 36, characterized in said agent is administered to a human patient in an amount of 1,4 to 285 mg per day.

38. Use according to one of the claims 1 to 37, characterized in that said creatine analogue has the general formula:

$$Z_1 \equiv\equiv C(\equiv\equiv Z_2) \equiv\equiv X\text{-}A\text{-}Y$$

and pharmaceutically acceptable salts thereof, wherein:

(a) Y is selected from the group consisting of: $-CO_2H$, -NHOH, $-NO_2$, $-SO_3H$, $-C(=O)NHSO_2J$ and $-P(=O)(OH)(OJ)$, wherein J is selected from the group consisting of: hydrogen, $C_1$-$C_6$ straight chain alkyl, $C_3$-$C_6$ branched alkyl, $C_2$-$C_6$ straight alkenyl, $C_3$-$C_6$ branched alkenyl and aryl;

(b) A is selected from the group consisting of: C, CH, $C_1$-$C_5$ alkyl, $C_2$-$C_5$ alkenyl, $C_2$-$C_5$ alkynyl, and $C_1$-$C_5$ alkoyl chain, each having 0-2 substituents which are selected independently from the group consisting of:

(1) K, where K is selected from the group consisting of: $C_1$-$C_6$ straight alkyl, $C_2$-$C_6$ straight alkenyl, $C_1$-$C_6$ straight alkoyl, $C_3$-$C_6$ branched alkyl, $C_3$-$C_6$ branched alkenyl, $C_4$-$C_6$ branched alkoyl, K having 0-2 substituents independently selected from the group consisting of: bromo, chloro, epoxy and acetoxy;

(2) an aryl group selected from the group consisting of: a 1-2 ring carbocycle and a 1-2 ring heterocycle, wherein the aryl group contains 0-2 substituents independently selected from the group consisting of: $-CH_2L$ and $-COCH_2L$ where L is independently selected from the group consisting of: bromo, chloro, epoxy and acetoxy; and

(3) -NH-M, wherein M is selected from the group consisting of: hydrogen, $C_1$-$C_4$ alkyl, $C_2$-$C_4$ alkenyl, $C_1$-$C_4$ alkoyl, $C_3$-$C_4$ branched alkyl, $C_3$-$C_4$ branched alkenyl, and $C_4$-$C_6$ branched alkoyl;

(c) X is selected from the group consisting of: $NR_1$, $CHR_1$, $CR_1$, O and S, wherein $R_1$ is selected from the group consisting of

(1) hydrogen;

(2) K where K is selected from the group consisting of: $C_1$-$C_6$ straight alkyl, $C_2$-$C_6$ straight alkenyl, $C_1$-$C_6$ straight alkoyl, $C_3$-$C_6$ branched alkyl, $C_3$-$C_6$ branched alkenyl, and $C_4$-$C_6$ branched alkoyl, K having 0-2 substituents independently selected from the group consisting of: bromo, chloro, epoxy and acetoxy;

(3) an aryl group selected from the group consisting of: a 1-2 ring carbocycle and a 1-2 ring heterocycle, wherein the aryl group contains 0-2 substituents independently selected from the group consisting of: $-CH_2L$ and $-COCH_2L$ where L is independently selected from the group consisting of: bromo, chloro, epoxy and acetoxy;

(4) a $C_5$-$C_9$ Alpha-amino-omega-methyl-omega-adenosyl-carboxylic acid attached via the omega-methyl carbon;

(5) a $C_5$-$C_9$ Alpha-amino-omega-aza-omega-methyl-omega-adenosylcarboxylic acid attached via the omega-methyl carbon; and

(6) a $C_5$-$C_9$ Alpha-amino-omega-thia-omega-methyl-omega-adenosylcarboxylic acid wherein A and X are connected by a single or double bond;

(d) $Z_1$ and $Z_2$ are chosen independently from the group consisting of: =O, $-NHR_2$, $-CH_2R_2$, $-NR_2OH$; wherein, $Z_1$ and $Z_2$ may not both be =O and wherein $R_2$ is selected from the group consisting of:

(1) hydrogen;

(2) K, where K is selected from the group consisting of: $C_1$-$C_6$ straight alkyl, $C_2$-$C_6$ straight alkenyl, $C_1$-$C_6$ straight alkoyl, $C_3$-$C_6$ branched alkyl, $C_3$-$C_6$ branched alkenyl, and $C_4$-$C_6$ branched alkoyl, K having 0-2 substituents independently selected from the group consisting of bromo, chloro, epoxy and acetoxy;

(3) an aryl group selected from the group consisting of: a 1-2 ring carbocycle and a 1-2 ring heterocycle, wherein the aryl group contains 0-2 substituents independently selected from the group consisting of:

$-CH_2L$ and $-COCH_2L$ where L is independently selected from the group consisting of: bromo, chloro, epoxy and acetoxy;

(4) a $C_4$-$C_8$ Alpha-amino-carboxylic acid attached via the omega-carbon;

(5) B, wherein B is selected from the group consisting of: $-CO_2H$, -NHOH, $-NO_2$, $-SO_3H$, $-C(=O)NHSO_2J$ and $-P(=O)(OH)(OJ)$, wherein J is selected from the group consisting of: hydrogen $C_1$-$C_6$ straight alkyl, $C_3$-$C_6$ branched alkyl, $C_2$-$C_6$ straight alkenyl, $C_3$-$C_6$ branched alkenyl and aryl; wherein B is optionally connected to the nitrogen via a linker selected from the group consisting of: $C_1$-$C_2$ alkyl, $C_2$ alkenyl, and $C_1$-$C_2$ alkoyl;

(6) -D-E, wherein D is selected from the group consisting of: $C_1$-$C_3$ straight chain alkyl, $C_3$ branched alkyl, $C_2$-$C_3$ straight alkenyl, $C_3$ branched alkenyl, $C_1$-$C_3$ straight alkoyl, and aryl; and E is selected from the group consisting of: $-(PO_3)_nNMP$, where n is 0-2 and NMP is a ribonucleotide monophosphate connected via the 5'-phosphate, 3'-phosphate or the aromatic ring of the base; $-[P(=O)(OCH_3)(O)]_m-Q$, wherein m is 0-3 and Q is a ribonucleoside connected via the ribose or the aromatic ring of the base; $-[P(=O)(OH)(CH_2)]_m-Q$, where m is 0-3 and Q is a ribonucleoside connected via the ribose of

the aromatic ring of the base; and an aryl group containing 0-3 substituents chosen independently from the group consisting of: Cl, Br, epoxy, acetoxy, -OG, -C(=O)G, and $-CO_2G$, where G is independently selected from the group consisting of: $C_1$-$C_6$ straight alkyl, $C_2$-$C_6$ straight alkenyl, $C_1$-$C_6$ straight alkoyl, $C_3$-$C_6$ branched alkyl, $C_1$-$C_6$ branched alkenyl, $C_4$-$C_6$ branched alkoyl; wherein E may be attached at any point to D, and if D is alkyl or alkenyl, D may be connected at either or both ends by an amide linkage; and

(7) -E, wherein E is selected from the group consisting of: $-(PO_3)_nNMP$, where n is 0-2 and NMP is a ribonucleotide monophosphate connected via the 5'-phosphate, 3'-phosphate or the aromatic ring of the base; $-P(P(=O)(OCH_3)(O))_m-Q$, wherein m is 0-3 and Q is a ribonucleoside connected via the ribose or the aromatic ring of the base; $-[P(=O)(OH)(CH_2)]_m-Q$, wherein m is 0-3 and Q is a ribonucleoside connected via the ribose of the aromatic ring of the base; and an aryl group containing 0-3 substituents chosen independently from the group consisting of: Cl, Br, epoxy, acetoxy, -OG, -C(=O)G, and $-CO_2G$, where G is independently selected from the group consisting of: $C_1$-$C_6$ straight alkyl, $C_2$-$C_6$ straight alkenyl, $C_1$-$C_6$ straight alkoyl; $C_3$-$C_6$ branched alkyl, $C_3$-$C_6$ branched alkenyl, $C_4$-$C_6$ branched alkoyl; and if E is aryl, E may be connected by an amide linkage;

(e) if $R_1$ and at least one $R_2$ group are present, $R_1$ may be connected by a single or double bond to an $R_2$ group to form a cycle of 5 to 7 members;

(f) is two $R_2$ groups are present, they may be connected by a single or double bond to form a cycle of 5 to 7 members; and

(g) if $R_1$ is present and $Z_1$ or $Z_2$ is selected from the group consisting of $-NHR_2$, $-CH_2R_2$ and $-NR_2OH$, then $R_1$ may be connected by a single or double bond to the carbon or nitrogen of either $Z_1$ or $Z_2$ to form a cycle of 4 to 7 members.

39. Use according to one of the claims 1 to 38, characterized in that said agent is used for the treatment of degenerative diseases of cartilage, in particular of arthritis.

40. A method for accelerating healing in an animal or human having a defect in bone or cartilage tissue caused by trauma, surgery or degenerative diseases, which method comprises administration of creatine compounds including analogues or pharmaceutically acceptable salts thereof or of creatine kinase.

41. A composition useful for the treatment of defects in bone or cartilage tissue of animals or humans caused by trauma or surgery, said composition comprising creatine compounds including analogues or pharmaceutically acceptable salts

thereof, characterized in that it is administered orally admixed with pharmacologically suitable carrier substances to improve bioavailability.

42. A composition according to claim 41, characterized in that the carrier substance is selected from the group of carbohydrates, in particular from maltodextrins and/or dextrose.

43. Use of creatine kinase, for the preparation of an agent for the treatment of bone or cartilage cells and tissues, characterized in that

a) bone or cartilage forming cells removed from a healthy individual or a patient are brought into cell culture and transfected with complementary DNA coding for creatine kinase isoforms and made to overexpress creatine kinase isoenzyme(s);

b) the metabolically engineered cells obtained in step a) are then expanded and cultivated to form in vitro genetically engineered cartilage or bone tissues transplantable into areas of cartilage or bone defects of said healthy individual or said patient.

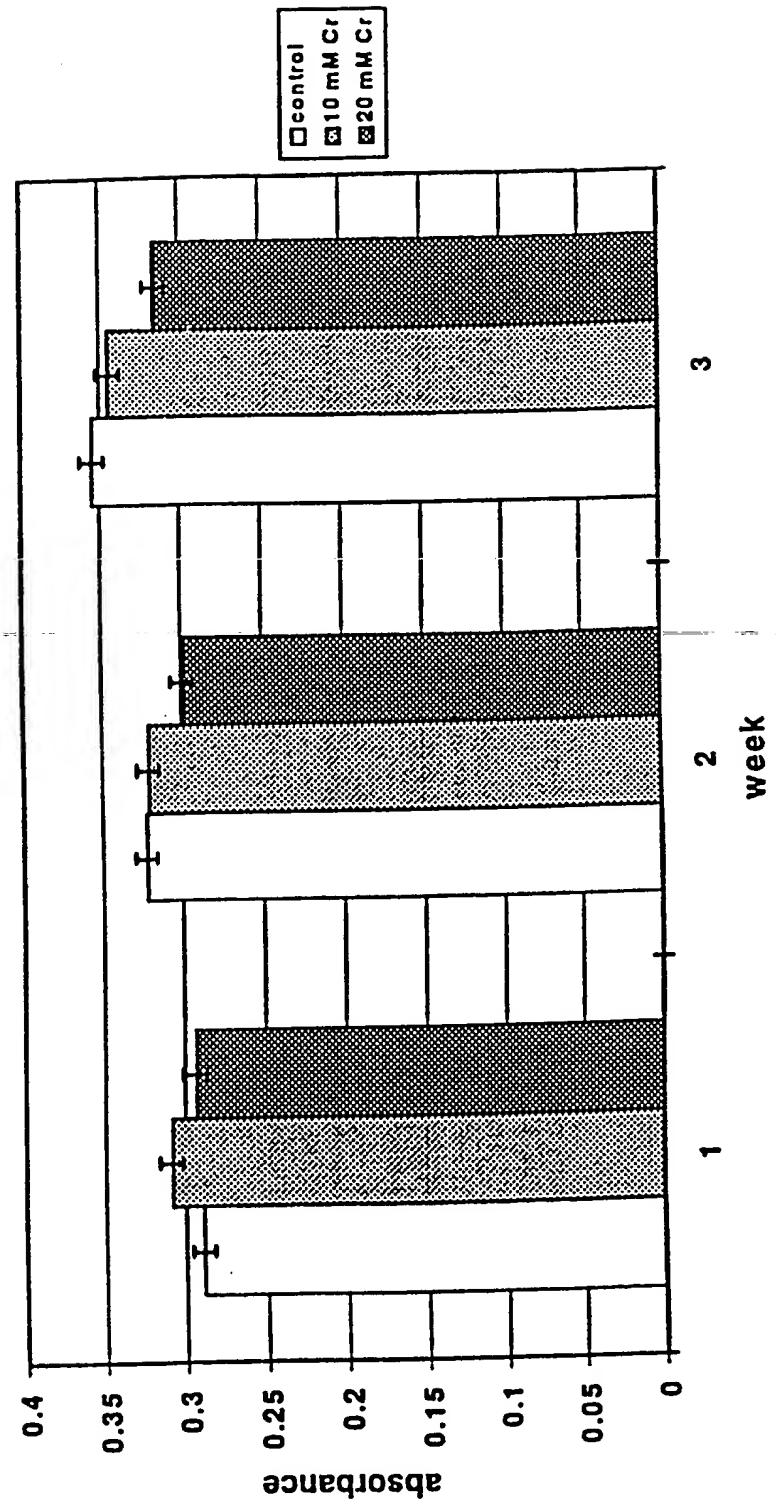



Fig.1

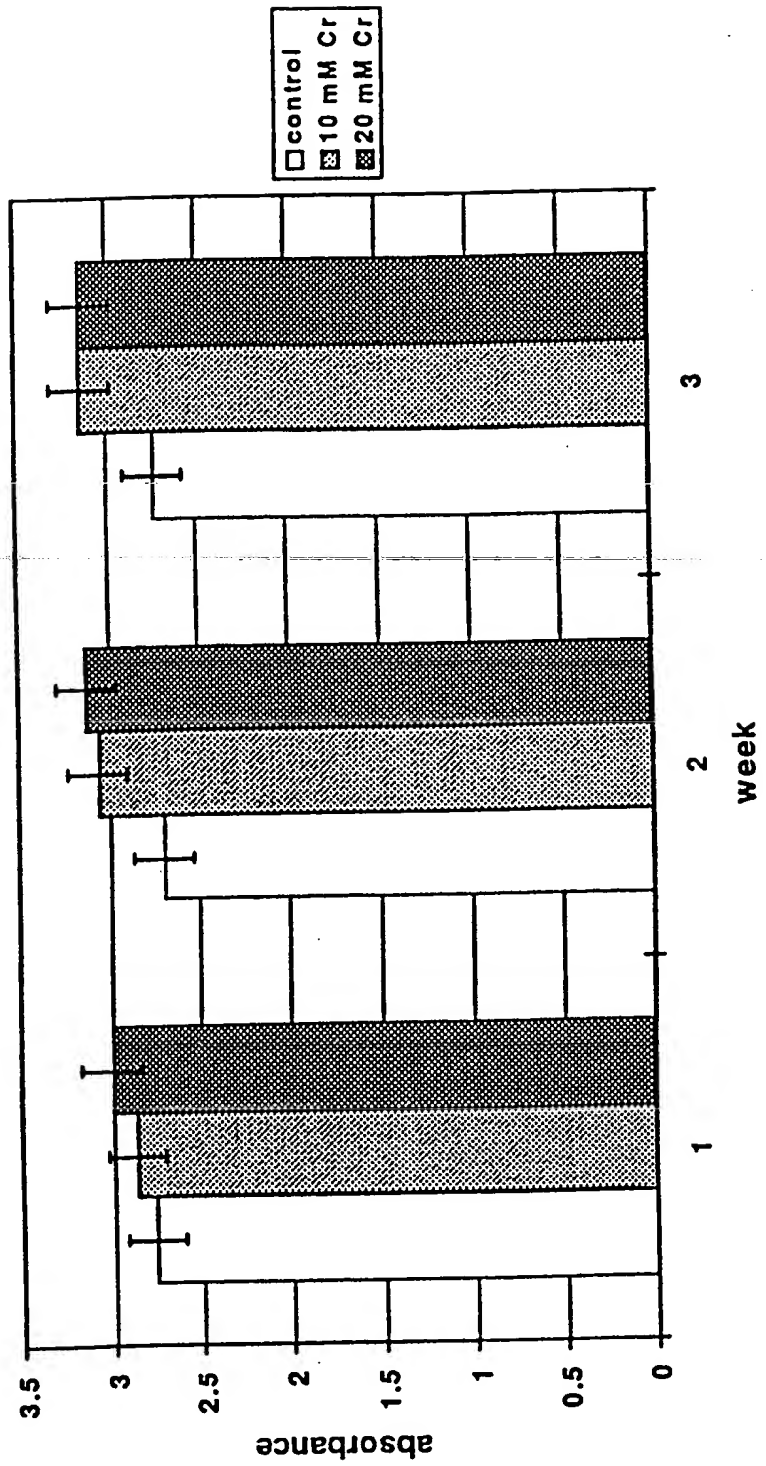



Fig. 2

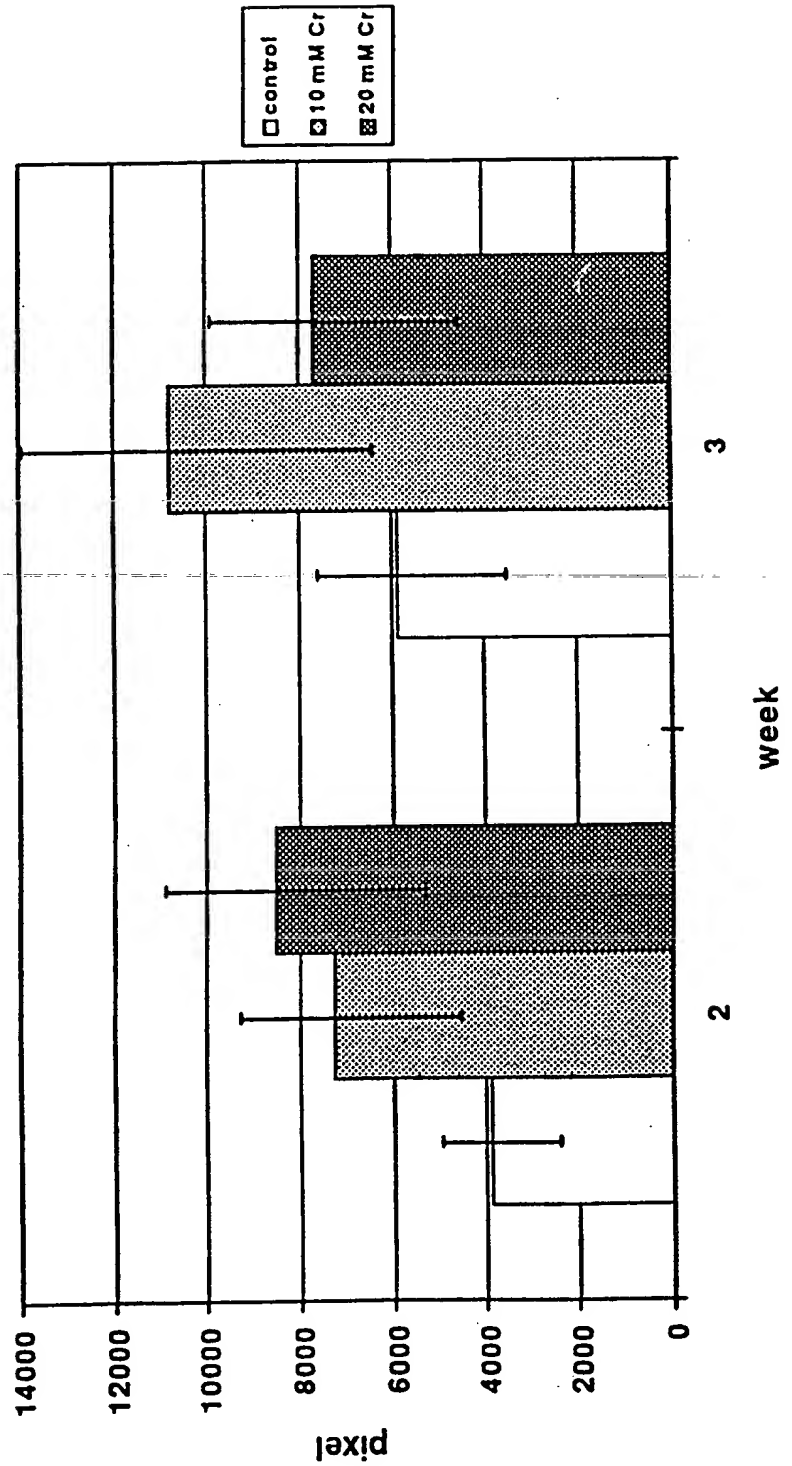

control
10 mM Cr
20 mM Cr
pixel
14000
12000
10000
8000
6000
4000
2000
0
week
2
3

Fig.3

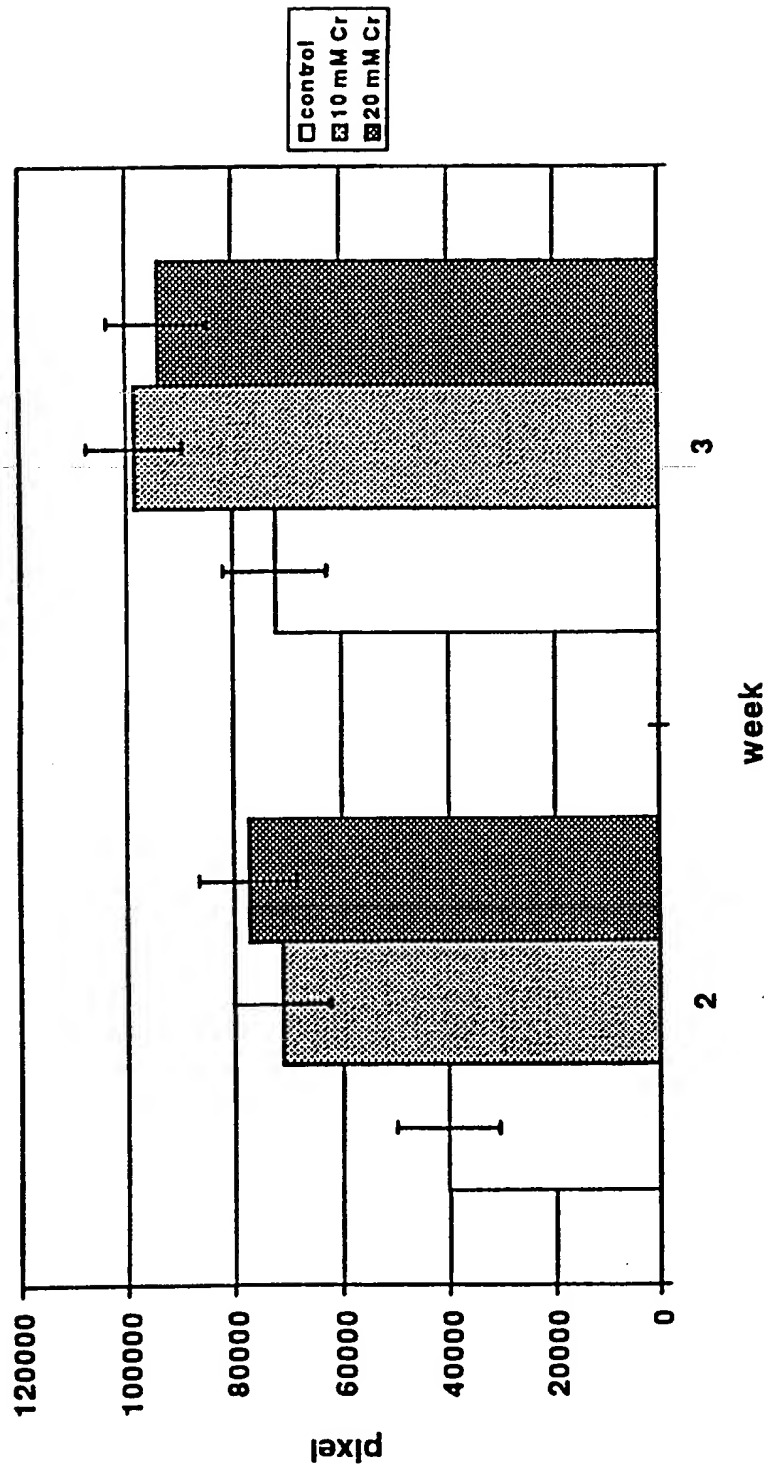

control
10 mM Cr
20 mM Cr
3
2
week
120000
100000
80000
60000
40000
20000
0
pixel

Fig. 4

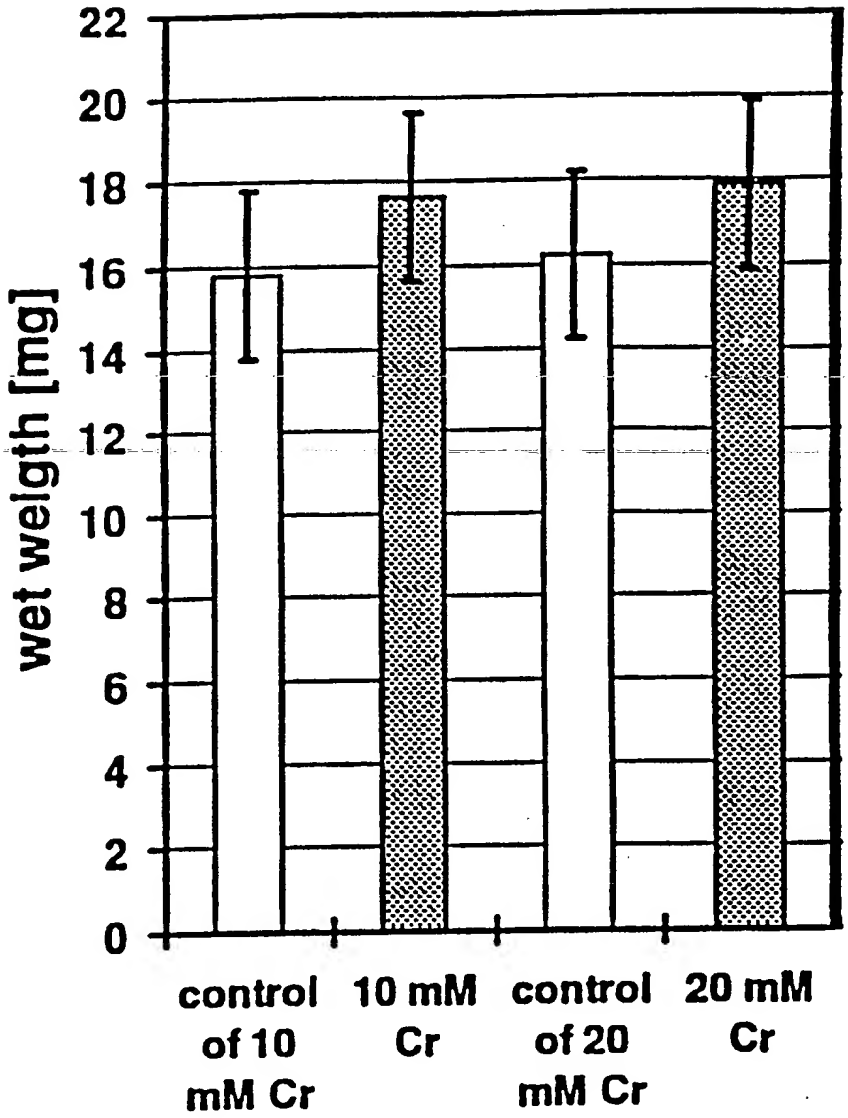



Fig. 5

# INTERNATIONAL SEARCH REPORT

Int[illegible]ional Application No
PCT/EP 98/04713

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/195 A61L27/00 A61L25/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | WO 97 45533 A (RUTHERFORD ROBERT BRUCE ;UNIV MICHIGAN (US); MOONEY DAVID J (US)) 4 December 1997<br>see page 2, line 20 - page 3, line 4<br>see page 15, line 21 - line 28<br>see page 65, line 20 - page 69, line 3<br>see page 82, line 1 - page 85, line 8<br>see page 87; table 6<br>---<br>-/-- | 1,2,5,7, 28,43 |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 19 March 1999 | 29/03/1999 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Cousins-Van Steen, G |

1

Form PCT/ISA/210 (second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/EP 98/04713

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | SOMJENSOMJEN D ET AL: "Nonhypercalcemic Analogs of Vitamin D Stimulate Creatine Kinase B Activity in Osteoblast-Like ROS 17/2.8 Cells and Up-regulate Their Responsiveness to Estrogens - new actions, new analogs, new therapeutic potential" STEROIDS: STRUCTURE, FUNCTION, AND REGULATION, vol. 63, no. 5-6, 6 May 1998, page 340-343 XP004132223 --- | |
| E | EP 0 891 719 A (NUTRICIA NV) 20 January 1999 see page 2, line 30 - line 33 see page 4, line 41 - line 57 see page 5, line 6 - line 12 see claims 4,10 ----- | 1,39 |

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/EP 98/04713

**Box I** Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claim 40 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the effects of the composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II** Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. ional Application No
PCT/EP 98/04713

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 9745533 A | 04-12-1997 | AU 3214797 A | 05-01-1998 |
| EP 0891719 A | 20-01-1999 | WO 9903365 A | 28-01-1999 |

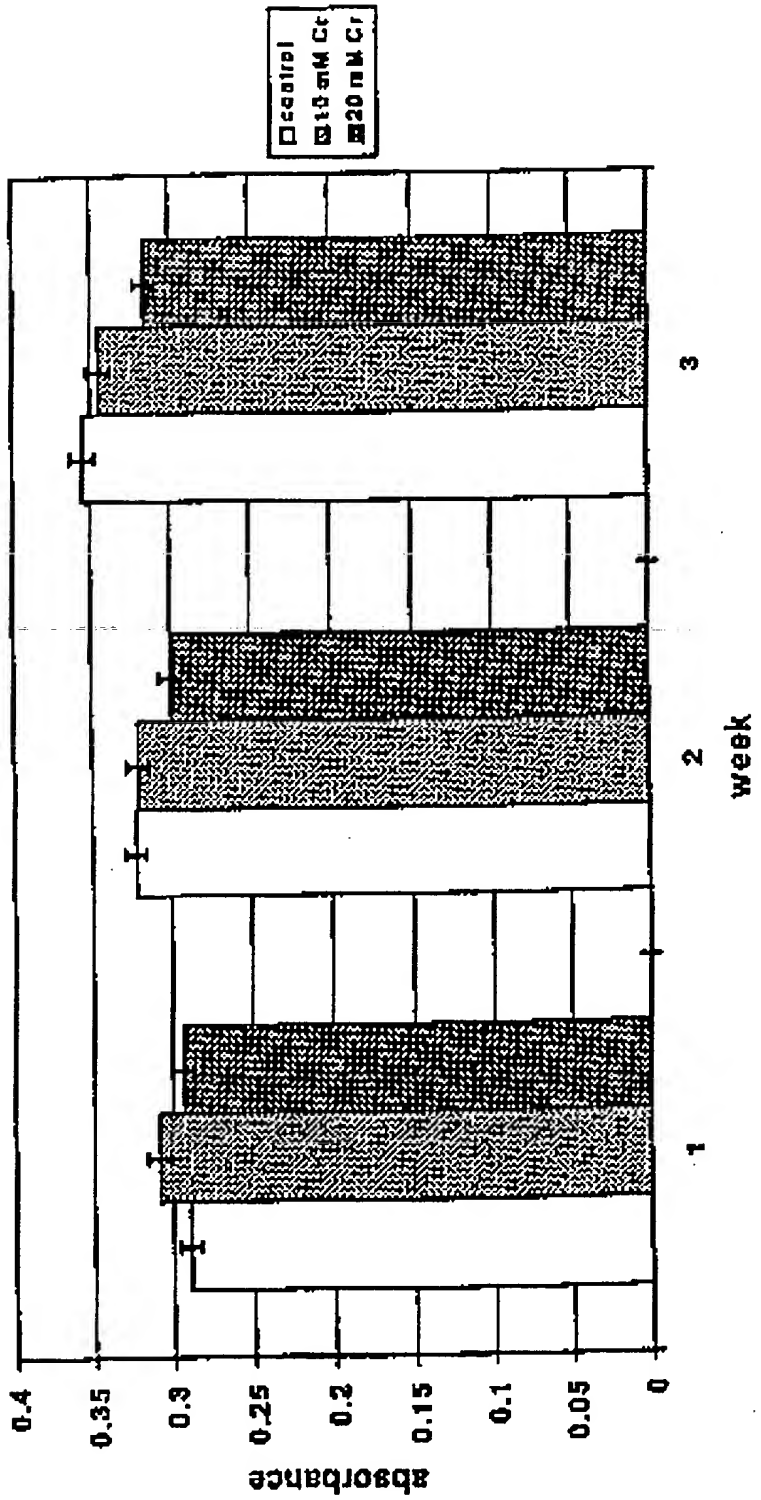

control
10 mM Cr
20 mM Cr
absorbance
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
week
1
2
3

Fig.1

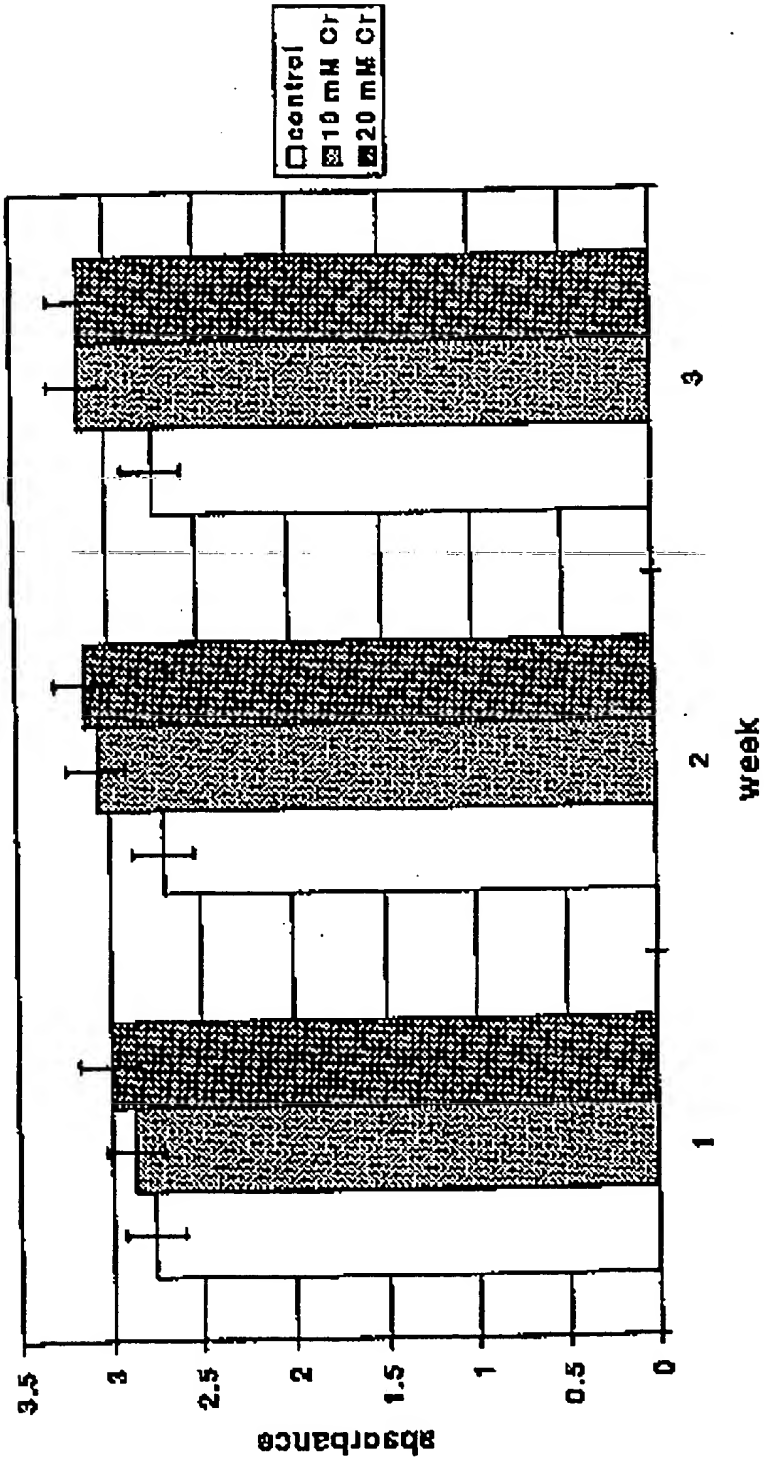



Fig. 2

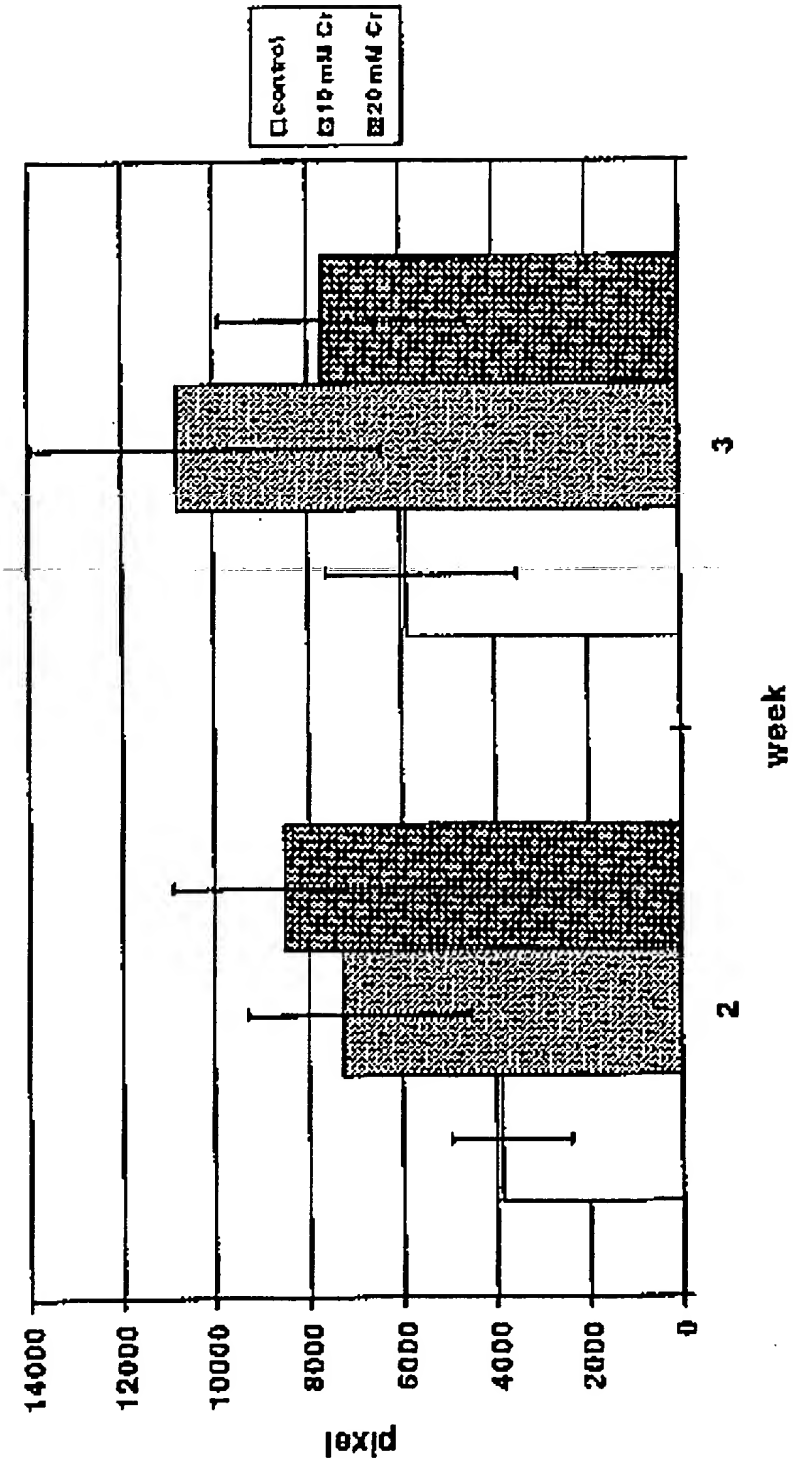

control
10 mM Cr
20 mM Cr
14000
12000
10000
8000
6000
4000
2000
0
pixel
2
3
week

Fig.3

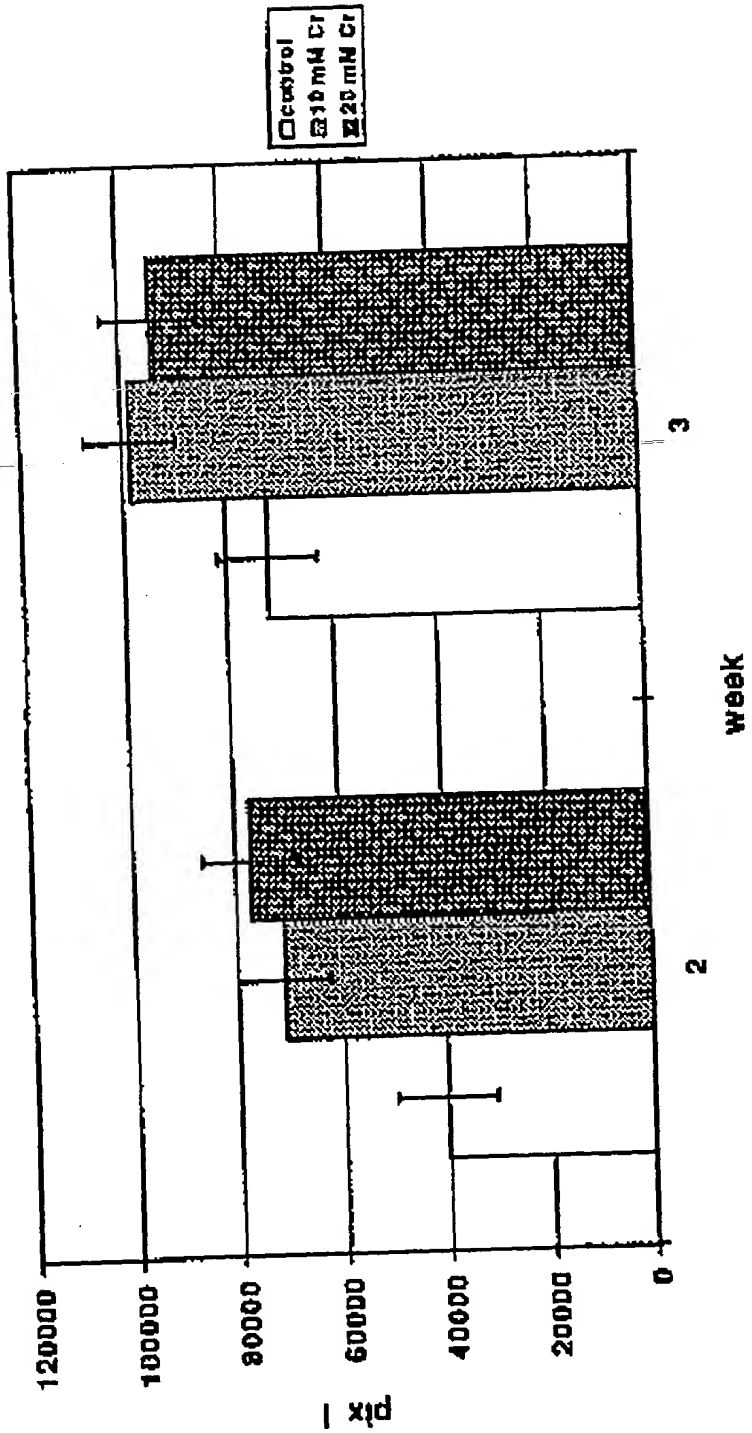

control
10 mM Cr
20 mM Cr
week
3
2
pix 1
120000
100000
80000
60000
40000
20000
0

Fig. 4

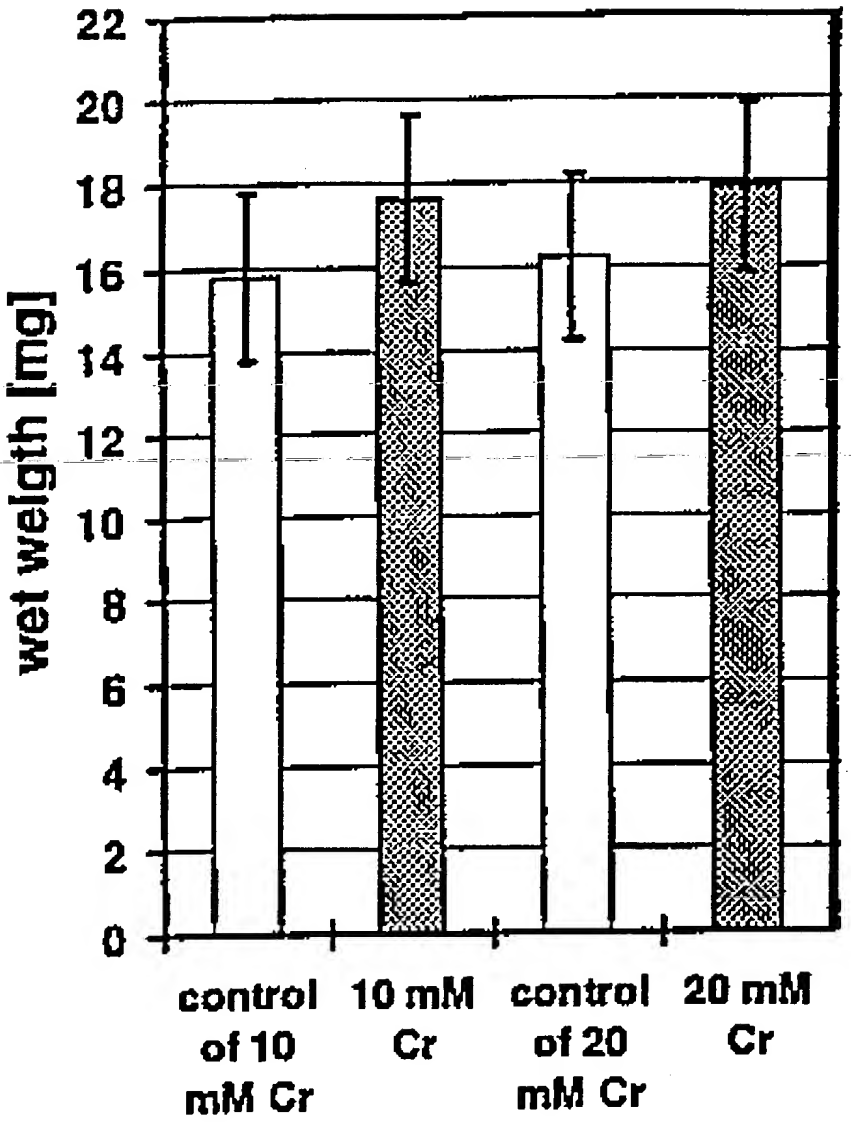



Fig. 5